GOVERNO DO ESTADO DE SÃO PAULO
SECRETARIA DE ESTADO DA SAÚDE
COORDENADORIA DE CIÊNCIA, TECNOLOGIA
E INSUMOS ESTRATÉGICOS DE SAÚDE
INSTITUTO BUTANTAN
SÃO PAULO, SP - BRASIL

**GOVERNO DO ESTADO
DE SÃO PAULO**

Memórias do Instituto Butantan

IX Reunião Científica Anual

IX Annual Scientific Meeting

Volume 64 - December 2007

São Paulo, SP – Brasil

2007

AUGUSTO ESTEVES

[1891–1966]

Aqui um fragmento da vida do Augusto, ou Sinhô como os irmãos e parentes próximos o chamavam, e Mané Coivara, como assinava suas poesias caipiras. Quarto filho de Geraldina Gomes de Oliveira Esteves e de Domingos José Esteves Júnior, nasceu em 16 de outubro de 1891 na cidade de São José da Boa Vista no Paraná. O primeiro aniversário foi comemorado em Avaré, outrora chamada Rio Novo. Bem menino começou a desenhar, pintar, desenhar... Não parou!

Aos 13 anos, vem para São Paulo acompanhando o irmão Lindopho que decidira ser Padre. Trabalhando como ajudante no comércio, estuda à noite e segue aprimorando–se no desenho e na pintura. Quatro anos depois, recebe a medalha de prata da Exposição do Centenário da Abertura dos Portos realizada no Rio de Janeiro. Motivado pelo prêmio, em troca dos serviços de limpeza do ateliê de Pedro Strina, pintor famoso por suas paisagens e retratos, recebia aulas de pintura do mestre.

Em 1912, conhece Vital Brazil no Instituto Butantan onde começa a trabalhar como desenhista, retratando com rara maestria as serpentes, e como ceroplasta, modelando peças anatômicas que reproduziam efeitos e seqüelas dos envenenamentos decorrentes de picadas de cobras... No Butantan conheceu Alvarina, segunda filha do cientista. E veio a paixão! E as filhas: Maria, Lygia, Jacy, Gláucia, Flávia e Itaé. Em 1919, segue para Niterói, Rio de Janeiro, acompanhando Vital Brazil na difícil missão de fundar o Instituto Vital Brazil.

Em 1934, retorna para São Paulo, indo trabalhar no Instituto Pinheiros e na Faculdade de Medicina da Universidade de São Paulo junto ao Departamento de Dermatologia. Em maio de 1980, foi inaugurado o Museu Augusto Esteves da Faculdade de Medicina da Universidade de São Paulo que apresenta várias reproduções em cera de lesões dermatológicas. Em abril de 2007, inaugurou-se a Sala Augusto Esteves com algumas de suas obras na Casa de Vital Brazil, museu na cidade de Campanha, Minas Gerais, local de nascimento do cientista.

Foi o responsável pela organização das comemorações do Centenário de nascimento de Vital Brazil em 1965. Ilustrou vários livros e muitos tantos trabalhos científicos; produziu textos, poesias e poemas, conversou e educou. Ensinou a versejar! Não parou...

Augusto é exemplo raro de que, como escreveu Mário de Andrade, a ciência como a arte é essencialmente social! Viveu levando adiante um imenso projeto de vida. Não parou...

Pelas criações,
Pela fidelidade à ciência,
Ao Vital de Campanha a dedicação,
Pelos versos certeiros do Mané Coivara,
Pela leve serenidade das mãos...
Pela assustadora justeza dos traços,
Pelos moldes revividos e desenhos expressivos,
Pelas cores transmitidas por seus olhos,
Pela constante inspiração transmitida,
A gratidão.

Osvaldo Augusto Brazil Esteves Sant'Anna

APOIO INSTITUCIONAL: INSTITUTO BUTANTAN e FUNDAÇÃO BUTANTAN, Financiamento desta publicação

Do volume 56 em diante, "Memórias do Instituto Butantan" passou a ser um relatório bienal das atividades científicas e técnicas do Instituto Butantan e, do volume 60, a conter os resumos da Reunião Científica Anual do Instituto Butantan. Na edição deste ano, volume 64, o "Memórias do Instituto Butantan" passa a ser apresentado na forma digital.

From Volume 56 on, "Memórias do Instituto Butantan" has been published as a biennial report of the scientific and technical activities of Instituto Butantan, and starting with Volume 60, the Abstracts of the Annual Scientific Meeting of Instituto Butantan were also included. Beginning this year, a digital form is substituting the hard-copy edition of the "Memórias do Instituto Butantan."

Endereço (Address) : Biblioteca, Instituto Butantan, Av. Vital Brazil 1500, 05503-900, São Paulo-SP, Brazil
Telefone: (Phone) +55 11 3726-7222, Ramal (Extension) 2129/2117
Fax : +55 11 3726-1505
E-mail : bibliobut@butantan.gov.br - www.butantan.gov.br

MEMÓRIAS do INSTITUTO BUTANTAN (Secretaria de Estado da Saúde).
São Paulo, SP, Brasil, 1918 - 1990, 52 (Supl. 1-3)
Em apenso, a partir de 1990, 52 (3) : BOLETIM DE BIOTECNOLOGIA.
1991, 53 (1) (Supl. 1, 2)
1992, 54 (1, 2)
1993, 55 (1, 2,) (Supl. 1)
1994-1995, interrompido
1996, 56 bienal, nova forma
1998, 57 bienal
2001, 58 bienal
2002, 59 bienal
2003, 60 anual
2005, 61 anual
2005, 62 anual
2006, 63 anual
2007, 64 anual CD-ROM

ISSN 1982-3045

EDITORIAL

In its 64th volume, **"Memórias do Instituto Butantan"** continues to present an overview of the diverse activities carried out at Butantan Institute, based on the scientific program and abstracts of the IX Annual Scientific Meeting. This meeting, whose theme is "Scientific Research and Dissemination," is an initiative of the Division for Scientific Development, and it involves all the divisions of the Institute.

Through symposiums, discussions will be held on the importance of scientific dissemination for the popularization and spread of knowledge, as an instrument for social development and a means of giving back to society for the public's investment in research.

The event entails three symposiums, three poster sections, scientific awards in four categories (scientific initiation, FUNDAP, master's and doctoral) and a special section about the memories of Butantan Institute.

The 283 posters to be presented in the IX Annual Scientific Meeting are representative of the different research areas being developed in the Institution, such as: Venoms, toxins and envenomation; Microorganisms and vaccines; Genetics and immunoregulation; Biology of snakes, arachnids and amphibians; Animal care and veterinary diseases; Science education; and Cellular biology. The best posters presented by undergraduate, graduate, Master's and Ph.D. students will be awarded with Instituto Butantan Prize. On the whole, we hope this issue provides an impressive overview of the diverse activities being carried out at Butantan Institute in 2007.

Denise V. Tambourgi
Editor-in-Chief

Ana M. Moura da Silva
Head of the Division for Scientific Development

INSTITUTO BUTANTAN
Board of Counselors and Divisions

Director
Mercadante, Otávio Azevedo

Directing Council
Mercadante, Otávio Azevedo (President)
Buss, Paulo Marchiori
Camargo, Erney Plessmann de
Canter, Henrique Moisés
Guralnik, Hernan Chaimovich
Jatene, Adib Domingos
Kalil, Jorge
Moura da Silva, Ana Maria
Higashi, Hisako Gondo
Teixeira, Paulo Roberto
Travassos, Luiz Rodolpho R. G.

Scientific Division
Director
Moura da Silva, Ana Maria

Research Council
Chudzinski-Tavassi, Ana Marisa
De Franco, Marcelo
Furtado, Maria de Fátima Domingues
Ho, Paulo Lee
Lebrun, Ivo
Serrano, Solange Maria de Toledo
Tambourgi, Denise Vilarinho
Yamanoue, Norma

Bioindustrial Division
Director: Higashi, Hisako Gondo

Production Council
Akimura, Olga Kikue
Guidolin, Rosalvo
Moro, Ana Maria
Prado, Sally Muller Affonso
Tenório, Elisabeth Christina Nunes

Cultural Division
Director: Canter, Henrique Moisés

Cultural Council
Martins, Rosana
Piagge, Vânia Dalle
Prezoto, Benedito Carlos
Puorto, Giuseppe
Ribeiro, Orlando Garcia
Wen, Fan Hui

Organizing and Scientific Committee for the
IX Annual Scientific Meeting of Instituto Butantan

Denise Vilarinho Tambourgi - President
Fan Hui Wen, Vice- President
Angela Silva Barbosa
Fernanda Calheta Vieira Portaro
Giuseppe Puorto
Maria de Fátima Domingues Furtado
Mônica Valdyrce dos Anjos Lopes Ferreira
Rute Maria Gonçalves de Andrade
Suzana Cesar Gouveia Fernandes
Viviane Fongaro Botosso
Waldir Pereira Elias Jr
Yara Cury

Collaborators
Elaine Rodrigues
Gibran Moraes dos Santos
Henrique Moisés Canter
Kátia Akimi Omine
Liana Maria Moretti
Nadja Rocha Terra
Osvaldo A. B. E. Sant'Anna
Ricardo Braga de Souza
Sérgio Marcello Humantschuk
Teresa Eiko Kawabata
Tiago de Paula Oliveira
Vânia Dalle Piagge

Acknowledgments
Cultural Division
Administration Division
Engineering Division
Biological Museum Team
Informatics Section
Fundação Butantan

English Reviewer: Albert Leyva
www.english-enhanced.com (al_leyva@yahoo.com)

Financial Support
Instituto Butantan
Fundação Butantan

Sponsorship
CRISTÁLIA
PRODUTOS QUÍMICOS FARMACÊUTICOS LTDA.

IX Annual Scientific Meeting of Instituto Butantan

"Research and Scientific Divulging"

December 05 – 07, 2007

Scientific Program

Wednesday 05/12/2007

09:00 - 09:30	**Opening Session**
09:30 - 12:00	**Symposium I: Scientific dissemination as a means of giving back to society, for public's investment in research** Coordinator: Otávio Azevedo Mercadante Instituto Butantan
9:30 – 9:55	**100 years of Science Education, 100 years of José Reis** Glória Kreinz Núcleo José Reis de Divulgação Científica
9:55 – 10:20	**The Scientific Journalism in forefront of Science and Technology politics** Fabíola Imaculada de Oliveira Sociedade Brasileira para o Progresso da Ciência
10:20 – 10:50	**Coffee break**
10:50 – 11:15	**Scientific Education in Brazil: Evaluation and Perspectives** Ildeu de Castro Moreira Departamento de Popularização de Ciência e Tecnologia, Ministério de Ciência e Tecnologia
11:15 – 11:40	**Science Education: a challenge for Butantan Institute** Otávio Azevedo Mercadante Instituto Butantan
11:40 – 12:00	General discussion
12:00 - 13:00	**Lunch**
13:00 - 15:00	**Poster session I**
15:00 – 16:00	**Scientific Initiation Award** Coordinator: Olga Ibañez Laboratório de Imunogenética, Instituto Butantan
16:00 - 16:30	**Coffee break**
16:30 – 17:30	**FUNDAP Award** Coordinator: Ivo Lebrun Laboratório de Bioquímica e Biofísica, Instituto Butantan

Thursday 06/12/2007

09:00 - 12:00	**Symposium II: Science Education and Popularization** Coordinator: Henrique M. Canter Divisão de Desenvolvimento Cultural, Instituto Butantan
9:10 - 9:35	**Museums without mystery: science education and challenges** Giuseppe Puorto Museu Biológico, Instituto Butantan
9:35 – 10:00	**Teaching about the Amazon in the environmental context: the role of the scientist in the dialogue process with society** Peter Mann de Toledo Instituto Nacional de Pesquisas Espaciais
10:00 – 10:30	**Coffee break**
10:30 – 10:55	**Butantan in the Amazon: sharing knowledge with the community** Fan Hui Wen Laboratório de História/Hospital Vital Brazil, Instituto Butantan
10:55 – 11:20	***Eppur si muove*** Ennio Candotti Faculdade de Física, Universidade Federal do Espírito Santo
11:20 - 12:00	General discussion
12:00 - 13:00	**Lunch**
13:00 - 15:00	**Poster session II**
15:00 - 16:00	**Master Award** Coordinator: Ana Marisa Chudzinski Tavassi Laboratório de Bioquímica e Biofísica, Instituto Butantan
16:00 – 16:30	**Coffee break**
16:30 - 17:30	**Doctoral Award** Coordinator: Catarina Teixeira Laboratório de Farmacologia, Instituto Butantan

Friday 07/12/2007

09:00 - 12:00 **Symposium III: Science Education - from the laboratory to the public**
Coordinator: Tiago de Paula Oliveira
Instituto Butantan

9:10 – 9:35 **Communication for Butantan Institute: a universe of possibilities**
Tiago de Paula Oliveira
Instituto Butantan

9:35 – 10:00 **Science education challenges: the experience of Pesquisa Fapesp Journal**
Ricardo Gabas Zorzetto
Revista Pesquisa FAPESP

10:00 – 10:30 **Coffee break**

10:30 – 10:55 **Transforming scientific information in communication: challenges, learning and the relationship with the public**
Yara Cury
Laboratório de Fisiopatologia, Instituto Butantan

10:55 – 11:25 **Social Impacts of science education through mass vehicles**
Drauzio Varela
Centro de Pesquisa e Tecnologia, Universidade Paulista

11:30 – 12:00 General discussion

12:00 – 13:00 **Lunch**

13:00 – 15:00 **Poster session III**

15:00 – 16:00 **Photograph Award**

16:00 – 16:30 **History of the Science Education at Butantan Institute**
Alessandra Fernandes Bizerra - Instituto Butantan

16:30 - 17:30 **Students' Scientific Awards**

17:30 **Closing session**

Poster Sessions	December 5 Session I	December 6 Session II	December 7 Session III
1.Venoms, toxins and envenomations	1-26	-	27-55
2.Microorganisms and vaccines	1-21	22-41	42-62
3.Genetics and immunorregulation	-	-	1-17
4.Biology of snakes, arachnids and amphibians	-	1-14	15-33
5.Animal care and veterinary diseases	1-5	-	-
6. Science	-	-	1-8
7.Cellular biology	-	1-17	-
8.Others	-	1-25	-
9.PIBIC program	1-42	-	-
10. FUNDAP program		1-19	-

1. Venoms, toxins and envenomations

1.01 Analysis of the skin secretion in casque-headed tree-frogs

Marques-Porto R[1], Ono RK[1], Antoniazzi MM[1], Lopes-Ferreira M[2], Lima C[2], Boletini-Santos D[2], Faivovich J[3], Haddad C[3], Rodrigues MT[4], Jared C[1]
[1]Laboratório de Biologia Celular, [2]Laboratório Especial de Toxinologia Aplicada – CAT/CEPID, Instituto Butantan, SP, Brazil; [3]Departamento de Zoologia, Universidade Estadual Paulista, Rio Claro, SP, Brazil; [4]Departamento Zoologia, Instituto de Biociências, Universidade de São Paulo, SP, Brazil

Introduction: Some species of anurans are characterized by the integumentary co-ossification of the head, which consists in the deposition of bone in the dermal layer of the skin. The group of casque-headed frogs has long been linked to phragmosis, a protective behavior in which the animal enters a hole and closes it with the head. Although cranial co-ossification in amphibians has frequently been associated with water economy, recent studies suggested that it can also function as a protection against predators and can be associated with different types of skin secretion exclusively present in the head. The group of casque-headed frogs presently forms a distinct clade (Tribe Lophiohylini) comprising the genera *Phyllodytes*, *Itapotihyla*, *Osteopilus*, *Osteocephalus, Tepuihyla*, *Corythomantis*, *Argenteohyla*, *Aparasphenodon*, *Nyctimantis* and *Trachycephalus.* The group is quite diverse as regards to its skull morphology, with some of them having heavily co-ossified skulls while others having no noticeable modification. **Objective:** The goal of this project was the comparison of electrophoretic profiles of body and head secretions of specimens of this clade having different skull morphologies. Profiles of *Itapotihyla langsdorfii* (Il), *Trachycephalus venulosus* (Tv) (two species without skull modifications), *Corythomantis greeningi* (Cg) and *Aparasphenodon brunoi* (Ab) (two species with heavily co-ossified skulls) were compared. **Methods:** Skin secretions from the body and head of the four species were submitted to SDS-polyacrylamide gel electrophoresis (PAGE) in 12% gels or 4 to 20% polyacrylamide gradient gels, and silver-stained. To verify differences in composition between head and body skin secretions, we compared the electrophoretic profiles. **Results:** All four species showed differences between head and body secretions. In both *C. greeningi* and *A. brunoi*, the only species studied with a well- characterized phragmotic behavior, the body secretions were considerably richer in bands than the head secretions, and *A. brunoi*, which is the most phragmotic of the two, was the clearest in this respect, with several bands appearing only in the body secretion. Additionally, *A. brunoi* is also the most secretory of the four species. *T. venulosus* showed very similar head and body secretions with characteristic bands exclusive to the head. *I. langdorfii*, on the other hand, is the only species with a more secretory head than body. **Discussion:** In the current phylogenetic hypotheses of the casque-headed tree frogs, *A. brunoi* and *C. greeningi* are the most closely related species of the four studied in this work, followed by *T. venulosus* and, more distantly, *I. langsdorfii*. While the taxonomic distribution of the secretions requires further study among other members of the tribe, and band homology needs to be carefully assessed, the overall patterns of band distribution seem to be congruent with current hypotheses in that both the head and body secretions of *C. greeningi* and *A. brunoi* share more bands between them than with the other two species. In turn, *T venulosus* shares more bands with these two than with *I. langsdorfii*.

Supported by CNPq, FAPESP, Fundação Butantan

1.02 Biological effect-driven biochemical assessment of the skin secretion of the tree frog *Phyllomedusa hypochondrialis*

Conceição K[1], Konno K[1], Melo RL[1], Lima C[1], Bruni FM[1], Pareja-Santos A[1], Antoniazzi MM[2], Jared C[2], Gilio JM[1], Silva CA[1], Conceição IM[3], Sciani J[3], Lopes-Ferreira M[1], Borella MI[4], Prezoto B[3], Daffre S[5], Camargo ACM[1], Pimenta DC[1]

[1]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, [2]Laboratório de Biologia Celular, [3]Laboratório de Farmacologia, Instituto Butantan, SP, Brazil; [4]Departamento de Biologia Celular e Desenvolvimento, [5]Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil

Introduction: Amphibian skin secretions contain several bioactive compounds, where peptides are a growing field of interest. However, few studies have been conducted in attempt to demonstrate the biological activities induced by the crude skin secretions of Phyllomedusa in murine models. **Objectives:** Our work focused on the depiction of the main physiopathological events and on determining the pattern of the local acute inflammatory response in mammals elicited by the frog skin secretion, as well as the identification of novel bioactive peptides present in this secretion. **Methods**: Glandular secretions of *P. hypochondrialis* specimens were analyzed with regard to nociceptive, edematogenic and vascular permeability activities, as well the inflammatory effect, in the mouse footpad, including leukocyte recruitment to local tissue from the peripheral blood. We also investigated the release of the cytokines IL-1, IL-6 and TNF-α, chemokines KC and MCP-1 and the eicosanoids LTB 4 and PGE(2) in mice. Furthermore, the skin secretion was fractionated by RP-HPLC and analyzed by MALDI-TOF/MS. Peptides were sequenced de novo by ESI-Q-TOF-MS/MS and synthesized through Fmoc-strategy. Bradykinin potentiation assays were performed on guinea pig ileum. **Results and Discussion:** Complex toxic effects of the *P. hypochondrialis* skin secretion and proposed mechanisms of action are presented and supported by *in vivo* data. The inflammatory reaction in the mouse footpad can be described as the release of IL-1, IL-6, TNF-alpha, KC, MCP-1, LTB 4 and PGE(2), corroborating the findings that crude skin secretion is able to induce local inflammation. Moreover, amphibian antimicrobial peptides, although well known, can still serve as scaffolds for antibiotic-resistant bacteria. Two antimicrobial peptides were screened, isolated and sequenced from the crude skin secretion. These peptides were effective against pathogenic bacteria, demonstrating no hemolytic activity, and could be classified as a Phylloseptin and a Dermaseptin. These peptides were sequenced by mass spectrometry and had their sequences chemically confirmed. Furthermore, in the present study, we also describe the isolation and biological characterization of a novel bradykinin potentiating-peptide (BPP) and three vasoactive peptides isolated from this skin secretion. The new BPP, named Phypo Xa is able to potentiate bradykinin activities *in vivo* and *in vitro*, as well as efficiently and competitively inhibit ACE and interact with the ACE testicular isoform. This is the first canonical BPP (i.e. Pyr-Aaa(n)-Gln-Ile-Pro-Pro) to be found not only in the frog skin but also in any other natural source other than snake venoms. All these findings are noteworthy for they not only present a series of new properties and molecules to be explored from frog skin secretion, but also demonstrate the efficiency of the biological effect-driven biochemical and proteomic approach to the finding of new molecules.

Supported by FAPESP, CNPq, CAPES

1.03 Myotoxicity induced by the venom of *Philodryas patagoniensis* (Serpentes: Colubridae)

Hess PL[1,2], Furtado MFD[1]
[1]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil; [2]Instituto de Biociências, Universidade de São Paulo, SP, Brazil

Introduction: Snake venoms are known to demonstrate considerable variability in their composition and biological activities, where proteolytic enzymes are the major components. Several of these have also been detected in venoms of the snakes belonging to the family Colubridae, and could have an important role in the development of the local damage caused by these venoms. In the family Colubridae, the venom of *Philodryas patagoniensis* produces local effects similar to the botropic accidents, such as: dermonecrosis, myonecrosis, pain, edema and hemorrhage, but in spite of such similarity, little is known about the importance of the myotoxicity in the way of action of secretions produced by Duvernoy's glands of the Colubridae snakes and about the action of components such as proteinases, in the induction of muscle damage. **Objectives:** The aim of this work was to characterize the myotoxicity induced by the venom of *P. patagoniensis* in skeletal muscle of mice, as well as to investigate the participation of proteinases. **Methods:** Groups of mice were injected in the right gastrocnemius with 30µg of the venom in 100µl of PBS (pH 7.2), and the animals of the control group just received PBS. At 15 min, 30 min, and 1, 3, 6 and 24 h after the injection, the muscles were removed and processed for the preparation of histological sections stained with hematoxylin-eosin. To investigate the participation of proteinases, mice were inoculated with the venom incubated with different inhibitors (EDTA, PMSF and 1-10-phenanthroline), for 30 min at room temperature. Three hours after the inoculation, the muscles were collected and processed as described above. **Results:** The histological analyses with light microscopy showed that the controls preserved the normal characteristics of skeletal muscle, while the injection of the venom produced effects such as: disorganization of the muscle fibers, hemorrhage, edema, prominent inflammatory infiltrate (PMN) and myonecrosis, beginning approximately 30 min after the inoculation and regressing six hours afterward. In the analysis of the muscles inoculated with the venom incubated previously with inhibitors, it was observed that EDTA was capable of inhibiting only hemorrhage, while inflammatory infiltrate, edema and myonecrosis persisted. Ophe and PMSF abolished myonecrosis, hemorrhage and inflammatory infiltrate, and only reduced edema and disorganization of muscle fibers. **Discussion:** These results suggest the participation of serine proteinases and metalloproteinases in the myotoxicity provoked by the *Philodryas patagoniensis* venom. However, the mechanism by which such proteinases induce the muscle damage is still not completely elucidated, requiring further biochemical and physiologic studies.

Supported by FAPESP

1.04 Characterization of the local action induced by the venom of *Philodryas patagoniensis* (Serpentes: Colubridae) in muscle cremaster of mice: an intravital microscopy study

Hess PL[1,2], Zychar B[3,4], Gonçalves LRC[4], Furtado MFD[1]

[1]Laboratório de Herpetologia, [4]Laboratório de Fisiopatologia, Instituto Butantan, SP, Brazil; [2]Instituto de Biociências, Universidade de São Paulo; [3]Departamento de Biotecnologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil

Introduction: The family Colubridae has the largest diversity, encompassing more than 65% of all of the existent snakes, but little is known about the venoms of members of this family, where the secretions produced by snakes belonging to the genus *Philodryas* are the most widely studied to date. In this genus, the secretion produced by *P. patagoniensis* is known to cause considerable local lesions, and envenomations, albeit infrequent, are characterized by pain, edema and hemorrhage, showing close similarity with the local signs displayed in envenomations by *Bothrops*. The effects of the venoms in tissues are extensively studied by histological, immunohistochemical and biochemical methods. However, the exact succession of the events that occur immediately after the contact between venom and tissue is not completely known. **Objectives:** Therefore, the present used the technique of intravital microscopy to analyze the effects of the topical application of the venom of *P. patagoniensis* on the microcirculatory network of the cremaster muscle. **Methods:** Anesthetized mice (23-27g) underwent surgical manipulation of the scrotum to expose the cremaster muscle, and they were placed on a plate mounted on the stage of a light microscope connected to a camera, a computer and a television monitor. The animals were observed 5, 10 and 15 min after the exposure of the microcirculation to the venom (0.1 and 0.5µg) or saline solution. **Results:** The administration of 0.1µg of the venom induced disturbances in postcapillary venules, characterized by vasodilatation (1min: 0.47±0.25; 3min: 3.16±1.28; 5min: 3.35±1.13; n = 5), hemorrhagic lesions distributed in foci (microbleedings) and alterations in the leukocyte-endothelial interactions, such as rolling and adhesion, which could indicate cellular recruitment, with decreased rolling and increased adhesion. The application of a higher dose (0.5µg) induced the same effects, however with different profiles. With this dose, the alterations in the diameter of the venules showed an initial transitory constriction followed by evident vasodilatation (1min: -0.44±0.25; 3min: 0.29±0.16; 5min: 0.90±0.32; n = 5) and the hemorrhagic lesions were shown to be continuous and no longer in foci. **Discussion:** The observed alterations indicate the inflammatory potential of this venom and the kinetics of the events that a complex interaction of several effects occurs during the development of the local effects and that such events can contribute to the severity of the envenomations.

Supported by FAPESP

1.05 Patagonfibrase, a hemorrhagic metalloproteinase from *Philodryas patagoniensis* snake venom, inhibits platelet adhesion and aggregation

Peichoto ME[1,2], Acosta O[1], Santoro ML[2]

[1]Faculdade de Ciências Veterinárias, Universidade Nacional do Nordeste, Argentina; [2]Laboratório de Fisiopatologia, Instituto Butantan, SP, Brazil

Introduction: Patagonfibrase is a 57.5-kDa hemorrhagic metalloproteinase isolated from the venom of the South American colubrid snake *Philodryas patagoniensis*, according to methods previously described. **Objective**: In the present study we aimed to evaluate the impact of patagonfibrase on platelets. **Methods and Results**: Intravenous administration of patagonfibrase to mice significantly prolonged the bleeding time (> 30 min; control: 3 min) as measured 1 hour after a bolus injection of the enzyme (15 μg). Therefore, patagonfibrase delays platelet-rich thrombus formation. In order to determine whether patagonfibrase affects platelet functions, adhesion and aggregation of washed platelets or platelet-rich plasma were tested in the presence of the enzyme. Adhesion assays performed in microplates coated with collagen showed that patagonfibrase inhibits platelet adhesion in a dose-dependent manner, and 50 % inhibition was obtained with 674 nM of the enzyme. Besides, patagonfibrase showed no platelet pro-aggregating activity *per se*, but it inhibited collagen-induced platelet aggregation, with an IC_{50} of 129 nM. Inactivation of the enzyme with Na_2EDTA produced no change in this inhibition. When 1 μg/ml collagen and 5 μg/ml patagonfibrase (final concentrations) were pre-incubated at 37 °C for 5 min, collagen could trigger platelet aggregation in the same way as adding the agonist after incubation of washed platelet suspensions with the enzyme. In addition, patagonfibrase exhibited 64 % inhibition of ADP-induced aggregation at a final concentration of 174 nM. This inhibition was enhanced to 93 % by pre-incubating the enzyme with fibrinogen at 37 °C for 2 min. Thrombin-, ristocetin-, convulxin- and A23187-induced platelet aggregations were not inhibited by patagonfibrase. **Discussion**: This is the first report on the characterization of an inhibitor of platelet adhesion and aggregation from the venom of a colubrid snake. The inhibitory activity on collagen-induced adhesion and aggregation may result from the binding of patagonfibrase to $\alpha_2\beta_1$ integrin. Regarding ADP-induced platelet aggregation, the enzyme likely inhibits aggregation by destroying intact fibrinogen and consequently by generating fibrinogen degradation products which act as competitive inhibitors of platelet-fibrinogen bridge formation. Due to its action on platelet functions, patagonfibrase may be used in studies on platelet physiology.

Supported by FAPESP, Fundação Butantan, CONICET

1.06 Local inflammation induced by *Philodryas olfersii* venom

Rocha MMT[1], Rodrigues E[1], Hess PL[1], Hebba CB[2], Farsky SHP[2], Furtado MFD[1]
[1]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil; [2]Faculdade de Farmácia e Bioquímica, Universidade de São Paulo, SP, Brazil

Introduction: Among the poisonous snakes of the family Colubridae, *Philodryas olfersii* induce a reaction in human victims characterized by substantial local effects consisting of pain, edema, hemorrhage, regional lymphadenopathy, erythema and chemosis. These effects are associated with important inflammatory reaction. **Objectives**: The objective of our work was to analyze the inflammatory action induced by *Philodryas olfersii* venom and the importance of catalytic activity and macrophages for its activity. **Methods**: Air pouches of 3.0 ml of sterile air were induced in the dorsal subcutaneous tissue of anesthetized mice. After seven days, the air pouches were reinforced and three days after the reinforcement (10 days after the initial induction) the venom in sterile PBS (5µg/500µl) was injected. Four hours after injection, the air pouches were washed with 3ml PBS and the exudate was collected. Control animals received only sterile PBS. Total cell counts were performed in Neubauer chambers. For differential cell counting, cytospin preparations were stained with Giemsa-May-Grünwald, and at least 300 cells were identified as polymorphonuclear leukocytes or mononuclear cells, based on conventional morphological criteria. To show the participation of metalloproteinases in cell migration, the venom was incubated 30 min at 37° with 5mM 1-10-phenanthroline or EDTA. After the injection, the exudate was collected and analyzed as described above. With the intravital microscopic method, we investigated the sequence of events induced by direct contact of *Philodryas olfersii* venom with the microcirculatory network in the cremaster muscle of mice. **Results**: The injection of 5µg of the venom of *Philodryas olfersii* into air pouches induced a marked leukocyte accumulation ($2.43 \times 10^6 \pm 0.16$ cells/ml), different ($p<0.05$) from control animals ($0.83 \times 10^6 \pm 0.08$ cells/ml). EDTA was not able to inhibit the cell migration significantly ($2.02 \times 10^6 \pm 0.16$ cells/ml), while the 1-10 phenanthroline inhibited 35% of the activity ($1.6 \times 10^6 \pm 0.08$ cells/ml). The topical application of the venom in the microcirculation evoked leukocyte-endothelial interactions determined by a decrease in the number of rolling leukocytes and an increase in the number of leukocytes adhered to endothelium, as well as the formation of hemorrhagic foci 30 and 60 min after the venom application. **Discussion**: The data show that the *P. olfersii* venom has an important inflammatory action, with metalloproteinase participation. The installation and the time-course of events, related to leukocyte-endothelial interactions and hemorrhagic lesions suggest a complex interaction of the effects that may contribute to the severity of the local lesion observed in *P. olfersii* envenomations.

1.07 Enzymatic and immunochemical characterization of *Bothriechis schlegelii* snake venom

Távora BCLF[1], Mejía NS[1], Lira MS[1], Suarez AM[3], Nuñez V[3], Marcelino JR[2], Otero R[3], Barbaro KC[1]

[1]Laboratório de Imunopatologia, [2]Seção de Plasma Hiperimunes, Instituto Butantan, SP, Brazil; [3]Grupo de Ofidismo/Escorpionismo, Universidade de Antióquia, Medellín, Colômbia

Introduction: The eyelash viper (*Bothriechis schlegelii)* is an arboreal snake that inhabits tropical and subtropical wet and moist forests from southern Mexico to Peru. Its name arises from the fact that the supraciliary scales form spine-like projections. A beige, green, yellow or grayish ground color is present in some populations, with markings ranging from tan to dark brown or black. *B. schlegelii*, together with *Bothrops* and *Porthidium* genera, is responsible for most reported snake bites in Colombia. The venom of this snake induces pain, local edema, mild hemorrhage and myotoxic activity. **Objectives:** The aim of this work was to characterize some enzymatic and immunochemical activities of *B. schlegelii* venom. **Methods:** SDS-PAGE (12.5%) was used to determine the electrophoretic pattern. Zymography was performed in a sodium dodecyl sulfate (12.5%) polyacrylamide electrophoresis gels to detect venom proteolytic and hyaluronidase activities of *B. schlegelii* venom, using fibrinogen (0.5 mg/ml), casein (2 mg/ml), gelatin (2 mg/ml) and hyaluronic acid (170 µg/ml) as substrates. ELISA and Western blotting, using antibothropic serum produced by Butantan Institute, were carried out to detect antigenic cross-reactivity. *Bothrops jararaca* venom was used as positive control. **Results:** *B. schlegelii* venom had low fibrinogenolytic activity with bands located in the 60 - 30 kDa region. However, intense enzymatic activity was detected when gelatin and casein were used as substrates with many different bands distributed between 80 – 30 kDa and 125 – 30 kDa, respectively. No hyaluronidase activity was observed. In SDS-PAGE, at least 11 bands were detected by silver nitrate, 7 of them major ones (between 203 - 30 kDa). Antibothropic serum recognized many components of *B. schlegelii* venom by ELISA. However, the titer obtained (160,000) was lower than that for *B. jararaca* venom (1,280,000) used as positive control. *B. schlegelii* venom components located between 80 - 30 kDa were recognized with the antibothropic serum by Western blotting. **Discussion:** The results suggest that *B. schlegelii* venom has little effect on the coagulation system and has enzymes that degrade extracellular matrix components that could explain, in part, the local damage observed in patients. Immunoassays showed antigenic cross-reactivity between *B. schlegelii* and *B. jararaca* venoms. Although, the titer was lower when compared with the positive control, the data still indicate that a few components of *B. schlegelii* venom have similar epitopes in relation to *B. jararaca* venom components.

Supported by FAPESP, Fundação Butantan, Antioquia University, CYTEP

1.08 *Bothrops atrox* and *Bothriopsis taeniata* snakes of the extreme Northwest of the Amazon region: immunogenicity and antigenic cross-reactivity of venoms

Furtado MFD[1], Travaglia–Cardoso SR[1], Soares OS[1], Pereira ASP[2], Fernandes DS[4], Marcelino JR[3], Tambourgi DV[2], Sant'Anna OA[2]

[1]Laboratório de Herpetologia, [2]Laboratório de Imunoquímica, [3]Serviço de Imunologia, Instituto Butantan, SP, Brazil; [4]Universidade Federal do Rio de Janeiro, RJ, Brazil

Introduction: Snakebites are still a critical public health problem in developing countries or isolated areas. In Brazil, the northern region has a high distribution coefficient worsened by the significant number of eventually unreported cases due to difficulties in access to health services, to the natural geographic barriers and the vast territory. In the extreme Northwest of Amazonia State, in the Rio Negro area, the species *Bothrops atrox*, *Bothrops brazili* and *Bothriopsis taeniata* are thought to account for the majority of snakebites in humans. It is known that venom constituents vary among individuals from the same species located at distinct regions and that these variations are clinically evidenced. The therapeutic anti-Bothropic serum produced at the Butantan Institute is obtained in horses immunized with a mixture of venoms from five species: *Bothrops jararaca, B. jararacussu, B. alternatus*, *B. moogeni* and *B. neuwiedi*. Therefore, this could compromise the potency of the anti-venom produced against the other *Bothrops* since, although there is a wide degree of epitopic cross–reactivity among the Viperidae species, there are peculiarities that should significantly intervene in the serum–neutralization efficacy. **Objectives and Methods:** The proposal of this study is the qualitative and quantitative evaluation of the antigenic cross-reactivity, the antigenic expression of toxins in *Bothrops* specimens and immunogenicity of the Amazon venomous species, and the comparison with the general venom mixture used for preparation of the heterologous therapeutic sera. These analyses were performed in genetically selected high responder mice [H_{III} line] which express no epitopic, nor idiotypic restrictions and have an accurate antigenic specificity recognition capacity. Groups of H_{III} mice were immunized with venom specimens from *B. atrox* from Rio Negro or from Maranhão State, with *Bothriopsis taeniata* venom or with the original Butantan mixture of *Bothrops* venoms. The individual antibody titers were determined by ELISA and the epitopic recognition by Western blotting. The lethal and minimal defibrinating and hemorrhagic doses were performed in vivo with the specific and polyvalent antiserums by neutralization assays. **Results and Discussion:** The Rio Negro *B. atrox* venom showed the highest immunogenicity and in contrast to the Maranhão specimen expressed higher complexity in antigenic varieties. In the *in vivo* assays, the defibrination action of *Bothriopsis taeniata* venom was absent but the lethal and hemorrhagic ones were more efficient than the *B. atrox* venom. These data are indicative of a speciation process related to the antigenic expression of toxins occurring in the *Bothrops* specimen's venoms and contribute to the general aspects that may guide the improvement of the anti–Bothropic therapeutic serum produced at Butantan. Analyses of the immunochemical and neutralization efficacies of new *Bothrops* venom combinations in the venom mixture, such as the substitution of *B. jararacussu* by *B. atrox,* are in progress.

Supported by FAPESP and CNPq

1.09 Neutralizing BaP1-induced hemorrhage and fibrinolytic activity with monoclonal antibodies

Assumpção GG[1], Silveira CRF[1], Tanjoni I[1], Faquim Mauro EL[1], Moura da Silva AM[1], Gutiérrez JM[2], Rucavado A[2], Takehara HA[1], Fernandes I[1]

[1]Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil; [2]Faculdade de Microbiologia, Instituto Clodomiro Picado, Universidade da Costa Rica, Costa Rica

Introduction: BaP1 is a 22.7-kD P-I class of snake venom metalloproteinases (SVMP) isolated from the venom of the snake *Bothrops asper*, a medically relevant species in Central America. This enzyme displays multiple tissue-damaging activities, including hemorrhage, myonecrosis, dermonecrosis, blistering, and edema, and thus, it plays an important role in the local tissue damage associated with *B. asper* envenomations. Four IgG MoAbs against BaP1 (MABaP1) recognizing only conformational epitopes were previously produced by our group. **Objective:** The aim of this study was to analyze the ability of MABaP1 to neutralize BaP1-induced hemorrhagic and fibrinolytic activity. **Methods:** Neutralization of hemorrhage induced by BaP1 was estimated by incubating 35 μg of BaP1 with protein A-affinity chromatography purified IgG of hybridoma supernatants (0.6:1 molar ratio) or with isotype control (monoclonal antibody anti-*Taenia crassiceps*), for 1 h at 37°C. After incubation, the mixture (100 μL) was centrifuged and injected i.d. into the abdomen of Swiss mice. The mice were killed 3 h after injection, the skin was removed and the area of the hemorrhagic spots was determined by multiplying the largest diameter by its perpendicular. Results are shown in $cm^2 \pm$ SD. Fribrinolytic activity of BaP1 was assayed by the fibrin-plate method. Briefly, a fibrin-agarose gel was prepared by mixing 1 mg/mL human fibrinogen with a preheated solution of 2% agarose in 50 mM Tris/HCl, pH 7.3 buffer containing 200 mM NaCl, 50 mM $CaCl_2$ and 2 U/mL thrombin. Samples of 10 μg BaP1 or BaP1, previously incubated with MABaP1 for 30 min at 37°C, were applied to wells of the solidified gel, incubated at 37°C for 18 h, and then the hydrolyzed area was measured. **Results:** The ability of the antibodies to neutralize BaP1-induced fibrinolytic activity was partial with clone 6 (67.7 ± 16.3) and high with clones 7, 8 and 3 (83.6 ± 14.2; 96.6 ± 5.9 and 100%, respectively). BaP1-induced hemorrhage was totally neutralized by clones 3, 6 and 8. MABaP1-7 not only did not inhibit but also could enhance hemorrhage under certain BaP1:MABaP1 ratios. **Discussion:** In conclusion, neutralizing MoAb against BaP1 conformational epitopes were obtained. X-ray crystallography of BaP1-MABaP1 complex is in progress and will help to elucidate the structure of the epitopes and the neutralization mechanisms. These results suggest that MABaP1 may be important tools to understand the role of P-I class SVMP in snakebite envenomation.

Supported by FAPESP

1.10 Comparative enzymatic and immunochemical studies between *Porthidium nasutum* and *Bothrops jararaca* snake venoms

Mejía NS[1], Távora BCLF[1], Lira MS[1], Suarez AM[3], Nuñez V[3], Marcelino JR[2], Otero R[3], Barbaro KC[1]

[1]Laboratório de Imunopatologia, [2]Seção de Plasma Hiperimunes, Instituto Butantan, SP, Brazil; [3]Grupo de Ofidismo e Escorpionismo, Universidade de Antióquia, Colombia

Introduction: The snake *Porthidium nasutum* (hog–nosed pit viper) inhabits tropical, wet and moist forests from southern Mexico to Ecuador. In Colombia, *Porthidium* and *Bothrops* genera are responsible for about 95% of snakebites. Envenomation by *Porthidium nasutum* causes mainly pain, edema, hemorrhage and necrosis at the site of the bite and, in a few cases, intracranial hemorrhage and acute renal failure. **Objectives:** The aim of this work was to compare some immunochemical and enzymatic activities between *P. nasutum* and *B. jararaca* venoms. **Methods:** Biochemical characterization was performed using polyacrylamide gel electrophoresis – sodium dodecyl sulfate (12.5%). Casein, gelatin, fibrinogen and hyaluronic acid were incorporated as substrates in SDS-PAGE (12.5%) to determinate the enzymatic activities. In addition, ELISA and Western blotting were performed to determin antigenic cross–reactivity between the two venoms using antibothropic serum produced by Butantan Institute. **Results:** The electrophoretic profile of *P. nasutum* venom showed at least 15 bands stained by silver nitrate, three of them with molecular weight similar to components of *B. jararaca* venom (50, 30 and 25 kDa). Zymography results demonstrated that *P. nasutum* venom had low fibrinogenolytic activity with a weak spot located around 35 - 30 kDa, whereas in *B. jararaca* venom, strong activity was observed between 60 – 30 kDa. Both gelatinolytic and caseinolytic activities were poorly detected in *P. nasutum* venom, with a single band located around 40 kDa and 35 kDa, respectively. However, *B. jararaca* venom showed intense gelatinolytic activity around 60 – 30 kDa and caseinases were detected between 90 – 30 kDa. Hyaluronidase activity was absent in both venoms. Similar antibody titers against *P. nasutum* (titer 640,000) and *B. jararaca* (titer 1,280,000) venoms were detected using antibothropic serum. This serum recognized at least 12 bands in the *P. nasutum* venom by Western blotting, the major ones being located between 70 – 30 kDa. In the case of *B. jararaca* (as expected since this venom is used in the immunization of horses), a strong reaction was observed with a large number of bands around 203 – 25 kDa. **Discussion:** Enzymatic activities of *P. nasutum* venom were less than those observed in *B. jararaca* venom. Furthermore, it was interesting to observe that antibodies present in antibothropic serum strongly recognize *P. nasutum* venom components. These results suggest that antibothropic serum produced in Brazil could neutralize the toxic activities of *P. nasutum* venom.

Supported by FAPESP, Fundação Butantan, Antioquia University, CYTED

1.11 Analysis of the antigenic properties and immunogenecity of Brazilian *Micrurus* snake venoms

Tanaka GD[1], Furtado MF[2], Sant'Anna OA[1], Tambourgi DV[1]
[1]Laboratório of Imunoquímica, [2]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: *Micrurus* bites can cause death by muscle paralysis and further respiratory arrest a few hours after envenomation and the general clinical manifestations are mainly neurotoxic symptoms. The elapid antivenom, produced by Instituto Butantan is obtained by hyperimmunization of horses with a mixture of *M frontalis* and *M. corallinus* venoms, which are snakes occurring in the South and Southeastern regions of Brazil. The existence of possible intra- and inter-specific variations in venom composition, associated with the geographic distribution of the snakes, can result in an inefficient neutralization of the lethal effects of the *Micrurus* venoms. **Objective:** The aim of this study was to analyze the antigenic properties and immunogenicity of nine *Micrurus* snake venoms**. Methods:** Mice from the high-antibody responder strain H_{III} were immunized by the subcutaneous route with venoms of nine species of the genus *Micrurus*, and serum was collected at different periods of time, after the completion of the immunization process. Antibody titers and pattern of antigen recognition were determined by ELISA and Western blotting, respectively. **Result:** Western blot and ELISA analyses showed that the venoms of *M. ibiboboca, M. lemniscatus, M. fulvius, M. altirostris, M. spixii, M. surinamensis, M. corallinus, M. frontalis* and *M. hemprichi,* were able to elicit antibody response in a high-antibody responder mouse strain H_{III}, although with different kinetic patterns and titers. It was also observed that the majority of the *Micrurus* antivenoms were capable of reacting, at different intensities, with the homologous and heterologous venoms. The anti-*M. surinamensis* and anti-*M. corallinus* sera*,* on the other hand*,* could only recognize the homologous venoms**. Discussion:** These results indicate that the *Micrurus* venoms display significant differences in their immunogenicity, as well as, in their antigenic properties, and these findings suggest that the production of serum for therapeutic use must be reviewed since the elapid antivenin currently produced could not fully neutralize the different *Micrurus* snake venoms occurring in Brazil.

Supported by FAPESP, Fundação Butantan, Antioquia University, CYTEP

1.12. Phospholipases A2 isolated from *Micrurus lemniscatus* coral snake venom induces apoptosis and Nitric Oxide alteration in rat neurons culture

Carvalho ND[1], Nunes CMB[1], Casais e Silva LL[3], Lebrun I[2], Afeche SC[1], Carneiro S[4], Sandoval MRL[1]

[1]Laboratório de Farmacologia, [2]Laboratório de Bioquímica e Biofísica, [4]Laboratório de Biologia Celular, Instituto Butantan, SP, Brazil; [3]Departamento de Fisiologia, Universidade Federal da Bahia, BA, Brazil

Introduction: The neurotoxicity of *Micrurus* venoms from the Americas has been poorly studied. **Objectives**: The aim of this work was to investigate the effects of two PLA2s, Ml-8 and Ml-9, isolated from the venom of *M. lemniscatus* in cultured hippocampal neurons. **Methods**: Neurons were dissociated from the hippocampi of E18 Wistar rat embryos. After treatment with trypsin and deoxyribonuclease, neurons were cultivated in B27-supplemented Neurobasal Medium. Cultures were kept at 37^0C in 5% CO_2 and plated at a density of 200 neurons/mm^2. After incubation of cultures (at day 7) with Ml-8 or Ml-9 (1000 ng/ml), neuronal cells were processed for electron microscopy. NO was determined by NOA 280 apparatus. **Results:** Treatment of the cells with Ml-08 and Ml-09 (1000 ng/ml) induced neuronal death mainly by apoptosis. The morphologic alterations observed by the electronic microscope were characterized by a condensation and fragmentation of the nucleus with the presence of chromatin granules, alterations in density and shape of the mitochondria and the presence of apoptotic bodies. Axonal and dendritic fragmentations were also observed. Treatment of the hippocampal cells for 24 or 48 h with 1000ng/ml Ml-08 induced an increase in NO levels. Ml-09 toxin treatment was not able to induce alteration in NO production. **Discussion**: Ml-8 and Ml-9 caused neuronal death mainly by apoptosis in cultured neurons. These data show the involvement of NO production in neuronal death induced by Ml-08 toxin. The toxicity may be associated with the binding of these toxins to specific brain sites and/or a β-BuTx–like effect at the pre-synaptic terminal. β-Bungarotoxin, the most often investigated pre-synaptic PLA2 neurotoxin from elapid venom applied to cultured rat hippocampal neurons, induced apoptotic cell death. These PLA2 can be a useful tool for exploring the death-signaling pathways of neurotoxicity.

Supported by FAPESP, Fundação Butantan

1.13 Relation between crotamine gene copy number and toxin concentration in the venom of *Crotalus durissus* rattlesnake

Oguiura N[1], Collares MA[1], Corrêa PG[2], Suzuki H[3], Furtado MFD[1]
[1]Laboratório de Herpetologia, [2]Laboratório de Influenza, [3]Laboratório de Biologia Celular, Instituto Butantan, SP, Brazil

Introduction: The *Crotalus durissus* venom shows an intraspecific variation, with some venoms containing crotamine (crotamine-positive) and others not (crotamine-negative). Among crotamine-positive venoms, we observed a variation in the crotamine concentration.(1) The crotamine-positive rattlesnake has a 1.8-kbp long crotamine gene, with three exons and two introns, and it was mapped to the end of the long arm of a chromosome.(2)Fluorescent *in situ* hybridization showed different intensities of signals between the two homologous chromosomes which could indicate a difference in gene copy number among the crotamine-positive rattlesnakes. **Objectives:** The aim of this work was to determine if there is a relation between gene copy number and the crotamine concentration in the venom. **Methods:** The crotamine concentration in the venom was determined by ELISA. Different dilutions of venom samples were adsorbed onto microtiter plates and an anti-crotamine antibody produced in rabbitd, followed by peroxidase conjugated-anti-rabbit IgG were used to detect crotamine(3). The gene copy number was determined by real time PCR, using the ABsolute™ QPCR SYBR Green Mix kit with 13 ng genomic DNA (estimated by fluorometry) and 700 nM of each primer, 15 min of polymerase activation at 95°C, followed by 30 cycles (30 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C). The primers were designed based on the cDNA sequence(4) and they amplify exon 2 of the crotamine gene. **Results:** The crotamine concentration in the venom varied from 5 to 26%, and the gene copy number in the haploid genome from 1 to 32 (n=13), and we found that there is a correlation (r=0.85). **Discussion:** Since we have found evidence of pseudogenes in some sequences, being inactive copies, they may have an influence on crotamine concentration. We should also consider other factors that change the expression efficiency such as mutation and methylation, and this point warrants further study to better understand toxin expression in snakes.

References: 1. Oguiura *et al.*, Toxicon 46: 363, 2005; 2. Rádis-Baptista *et al.*, Toxicon 42: 747, 20; 3. Oguiura *et al.*, Toxicon 38: 443, 2000; 4. Rádis-Baptista *et al.*, Toxicon 37: 973, 1999.
Supported by FAPESP, FUNDAP
Acknowledgements: Laboratório de Imunogenética for the use of PT 200 - Chroma 4 Continuous Fluorescence Detection, the Real Time PCR System from MJ Research, and Centro de Biotecnologia for the use of Original Multiskan EX from Labsystems

1.14 Cytotoxic effects of crotoxin on murine melanoma cells

Calvo F[1,2], Guzovsky E[1], Spencer PJ[2], Casare M[2],Vassão RC[1]
[1]Laboratório de Genética, Instituto Butantan, SP Brazil; [2]Centro de Biotecnologia, Instituto de Pesquisas Energéticas e Nucleares, CNEM, SP, Brazil

Introduction: Crotoxin is the most abundant and active toxin from the venom of the Brazilian rattlesnake *Crotalus durissus terrificus*. It has been described as a neurotoxin involved in pre-synaptic blockade of neuromuscular junctions. Some authors have also attributed to this toxin a direct cytotoxic effect, and have suggested its use as a therapeutic agent against tumor cells. **Objectives**: In the present work, we investigated the cytotoxicity of crotoxin in a murine melanoma cell line and in normal fibroblasts. **Methods**: Cells (5.4 x 10^5/ml) were grown in 96-well microplates. After 24 h, the cells were incubated with increasing concentrations of crotoxin, ranging from 12.5 to 400 μg/ml, previously diluted in RPMI with 10% calf serum. After 48 h, the toxin-containing medium was replaced with MTT in phosphate-buffer saline and incubated for 3 h. The formazan crystals were then solubilized with DMSO and the absorbance was read at 540 nm. **Results and Discussion**: Our results indicate that crotoxin is toxic to both cell strains, but the tumor cells appear to be more sensitive than the fibroblasts. Indeed, while 100 μg/ml of crotoxin was sufficient to abolish the cell viability of the melanoma cells, about 200 μg/ml were necessary to elicit the same effect in fibroblasts.

Supported by CNPq

1.15 Effect of *Crotalus durissus terrificus* snake venom and crotoxin on the viability of endothelial cells *in vitro*

Matsubara MH, Lima SA, Zamunér SF, Teixeira CFP
Laboratório de Farmacologia, Instituto Butantan, SP, Brazil

Introduction: *Crotalus durissus terrificus* venom (CdtV) induces systemic effects that interfere with both central nervous and coagulation systems. The main component of CdtV is crotoxin (CTX), which contains of two different subunits: crotapotin (CA) and phospholipase A_2 (CB). CTX exerts neurotoxic and myotoxic effects and inhibits pathophysiological processes such as inflammation and hyperalgesia. Despite the importance of endothelial cells (EC) in homeostasis, the effects of CdtV and CTX on these cells are still unknown. **Objectives:** In this study, the effects of CdtV and CTX on the viability of endothelial cells in culture were evaluated. **Methods:** EC from a murine endothelioma cell line (tEnd) were cultured in RPMI-1640 medium with 10% FBS and seeded onto 96-well microplates for formation of monolayers. After reaching confluence (48 h) EC monolayers were incubated with CdtV, CTX (1, 10 or 100 μg/mL) or RPMI (control). EC viability was evaluated by lactate dehydrogenase (LDH) and tetrazolium salt reduction (MTT) assays using selected incubation periods. **Results:** CdtV at the highest concentration significantly decreased the metabolic activities of endothelial cells by 35, 51, 89 and 82% at 1, 3, 24, 48 h of incubation, respectively. The lowest concentrations of CdtV did not affect this parameter. In addition, this venom at the highest concentration induced a significant release of LDH into the cell culture medium at 3 and up to 48 h (48 and 100%, respectively). In contrast, CTX reduced the EC metabolic activities only after 24 and 48 h of incubation by 10 and 43%, respectively, and at the highest concentration; the lowest concentrations of CTX did not modify EC metabolism. A significant release of LDH was detected 24 and 48 h after incubation of EC with the highest concentration of CTX. **Discussion:** These data show for the first time the ability of CdtV and CTX to affect the metabolism and viability of endothelial cells *in vitro*, CTX being less efficient than CdtV. These effects may have implications for the vasculature physiology with regard to the action of CdtV and CTX.

Supported by FAPESP, CNPq

1.16 Effects of *Crotalus durissus terrificus* snake venom (CdtV) and of a phospholipase A_2 isolated from this venom on the activity of neutral aminopeptidase APN/CD13 of murine macrophage

Olivo RA[1], Silveira PF[1], Soares AM[2], Teixeira CFP[1]
[1]Laboratório de Farmacologia, Instituto Butantan, SP, Brazil; [2]Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, SP, Brazil

Introduction: Neutral aminopeptidase APN/CD13 is an important enzyme in cellular adhesion processes and modulation of the activity of several inflammatory mediators released from macrophages (Mϕs), the main effector cells of the immune system. Under stimulation, these cells are activated by a process involving biochemical and functional changes. Alterations of macrophage enzymes by venoms and toxins represent a new field of research in toxinology. **Objectives:** In this study, the effects of both CdtV and a phospholipase A_2 (CB_2) isolated from this venom on the neutral aminopeptidase activity (APN/CD13) in soluble (S) and membrane-bound (M) fractions of murine Mϕs in culture were evaluated. **Methods:** The murine macrophage cell line RAW 267.4 was grown in RPMI medium with 10% FBS. Mϕs, $4x10^6$ cells/mL, were incubated with CdtV (3 μg/mL) or CB_2 (6.5 μg/mL) or RPMI (control) for 1 h. The APN/CD13 activity was measured by fluorogenic assay using a naphthylamide substrate. **Results:** CdtV and CB_2 increased APN/CD13 activity (720.1 ± 35.3 and 754.3 ± 32.4 UP/mg protein, respectively) in the soluble fraction of macrophages in comparison to control (481.2 ± 11.7 UP/mg protein), but did not affect this enzyme activity in the M fraction. **Discussion:** The results demonstrate that both CdtV and the phospholipase A_2 CB_2 exert a direct effect on macrophages, increasing the activity of APN/CD13 in the soluble fraction. Considering the high concentration of phospholipase A_2 in CdtV, we suggest that CB_2 is important in the CdtV stimulatory effect on Mϕ APN/CD13.

Supported by FAPESP

1.17 Effect of *Crotalus durissus terrificus* venom on phagocytosis by polymorphonuclears, evaluated by the model of peritonitis

Lima TS, Nunes FPB, Sampaio SC, Sousa-e-Silva MCC
Laboratório de Fisiopatologia, Instituto Butantan, SP, Brazil

Introduction: Previous work showed that *Crotalus durissus terrificus* snake venom (CdtV) modulates macrophage function, inhibiting the spreading and phagocytic activity of theses cells. Recently, CdtV was shown to inhibit carrageenan-induced inflammatory response. Despite these findings, the effect of CdtV on polymorphonuclear cell functions has not been determined. **Objectives:** The aim of this study was to investigate the effect of CdtV on phagocytosis activity, via C3b receptor, by polymorphonuclears obtained by carrageenan-induced peritonitis. **Methods:** Polymorphonuclears were obtained from the peritoneal cavity of male Wistar rats (170 g) 4 h after the intraperitoneal (i.p.) administration of carrageenan (cg) (4.5 mg/kg). Phagocytosis of opsonized zymosan was evaluated after treatment *in vitro* or *in vivo* with CdtV. For *in vitro* treatment, cells (1.2 x 10^6 cells/mL) were incubated with RPMI 1640 medium (control) or with RPMI 1640 medium containing CdtV (0.25, 0.5 and 1.0 μg/mL). For *in viv*o treatment, CdtV or sterile saline (control) was administered by subcutaneous (s.c.) route (0.18 mg/kg) to rats at different time periods: 2 h, or 1 or 4 days before the i.p. administration of cg (4.5 mg/kg). **Results:** *In vitro,* CdtV at different concentrations reduced significantly the phagocytic activity of polymorphonuclears: 0.25 μg/mL: 38% (cg+CdtV: 79.2 ± 3.9; $p<0.001$), 0.5μg/mL: 36% (cg+CdtV: 82.0 ± 4.4; $p<0.001$) and 1.0 μg/mL: 34% (cg+CdtV: 84.4 ± 5.1; $p<0.001$) when compared to the control (cg+medium: 127.4±10; $p<0.001$). The injection of CdtV also reduced the percentage of phagocytosis: 2 h: 42% (cg+CdtV: 96.6 ± 2.4; cg+saline: 166.6 ± 17.7; $p<0.001$), 1 day: 43% (cg+CdtV: 90.2 ± 5.7; cg+saline: 156.2 ± 12.1; $p<0.001$) and 4 days: 51% (cg+CdtV: 75 ± 8.4; cg+saline: 151.8 ± 7.8; $p<0.001$). **Discussion:** The results show that CdtV inhibits the phagocytic activity of polymorphonuclears both *in vitro* and *in vivo*. Thus, taking into consideration the role of polymorphonuclears in the inflammatory response, the effect of CdtV on phagocytosis by polymorphonuclears can contribute to explaining its inhibitory effect on the inflammatory response induced by cg.

Supported by FAPESP

1.18 Contribution of crotoxin (ctx) on anti-inflammatory effect of *Crotalus durissus terrificus* snake venom (CdtV)

Nunes FPB, Lima TS, Zychar BC, Sousa-e-Silva MCC
Laboratório de Fisiopatologia, Instituto Butantan, SP, Brazil

Introduction: Recently, we demonstrated that CdtV demonstrates a long-lasting anti-inflammatory effect. This venom inhibits paw edema and cell migration to the peritoneal cavity induced by carrageenan (cg). **Objectives:** The aim of this study was to investigate the role of crotoxin, the main toxin of this venom, in the development of paw edema, cell migration to the peritoneal cavity and leukocyte-endothelium interaction in the microcirculation of the cremaster muscle induced by carrageenan. **Methods:** Crotoxin (ctx 0.89 μg/50 μL s.c.) or saline (sal 50 μL) was injected in male Swiss mice (18-22g) (n=6), 1 h before or after intraplantar injection of carrageenan (300 μg/50 μL) or saline (contralateral paw) (50 μL). Paw edema was evaluated after different periods of time using a pachymeter. Cell migration was evaluated 4 h after intraperitoneal injection of carrageenan (300 μg/200 μL) or saline (200 μL) in the animals treated with ctx (0.89 μg/50 μL s.c.) or saline. The number of total peritoneal cells was determined in a Neubauer hemocytometer, and differential counts were performed in smears stained with panchromatic stain. To analyze leukocyte-endothelium interaction in the microcirculation of the cremaster muscle, carrageenan (300 μg/100 μL) or saline (100 μL) were injected into the scrotum of animals 1 h before or after ctx (0.89 μg/50 μL s.c) or saline (50 μL) injection. Leukocytes rolling on the endothelial surface and migrating to the extravascular tissue were counted by intravital microscopy 1 h after carrageenan or saline injection. **Results:** Pre and post-treatments with ctx reduced paw edema, cell migration and leukocyte-endothelium interaction induced by carrageenan when compared with respective controls, at all time intervals evaluated. The reduction of paw edema was higher at 72 h after carrageenan injection. At this time, the reduction was 33% (ctx + cg: 18.77 ± 1.49; sal + cg: 28.00 ± 1.76; $p<0.05$) for pre-treatment and 30% (cg + ctx: 22.52 ± 1.49; cg + sal: 28.00 ± 1.76, $p<0.05$) for post-treatment. The inhibition of cell migration was 38% (ctx + cg: 0.89 ± 0.9; sal + cg: 1.43 ± 0.12; $p<0.05$) for pre-treatment and 34% (ctx + cg: 0.85 ± 0.06; sal + cg: 1.28 ± 0.10; $p<0.05$) for post-treatment. The treatment with CdtV 1h before or after carrageenan injection (300 μg/100 μL) into the scrotum induced a decrease in cell adhesion to the endothelium surface (pre-treatment: ctx + cg: 1.22 ± 1.92; sal + cg: 6.4 ± 0.89; $p<0.05$; post-treatment: cg + ctx: 8.5 ± 0.42; cg + sal: 13.4 ± 0.74; $p<0.05$) and cell migration to the extravascular tissue (pre-treatment: ctx + cg: 1.2 ± 1.09; sal + cg: 5.4 ± 0.89; $p<0.05$; post-treatment: cg + ctx: 2.2 ± 0.66; cg + sal: 5.2 ± 0.48; $p<0.05$). **Discussion:** The results of this study showed that crotoxin inhibited the development of paw edema, cell migration and leukocyte-endothelium interaction induced by carrageenan in the microcirculation of cremaster muscle. It is concluded that the crotoxin, the main toxin of *Crotalus durissus terrificus* snake venom, could contribute to the anti-inflammatory effect of this venom.

Supported by CNPq

1.19 Sex differences in the pain threshold and in the antinociceptive effect of Crotalphine, an opioid analgesic obtained from C*rotalus durissus terrificus* snake venom (CdtV)

Britto L[1], Picolo G[1], Anselmo-Franci JA[2], Cury Y[1]
[1]Laboratório de Fisiopatologia, Instituto Butantan, SP, Brazil; [2]Faculdade de Odontologia de Ribeirão Preto, Universidade de São Paulo, SP, Brazil

Introduction: Sex differences in pain sensation and in the response to analgesics, particularly opioids, have been described for humans and animals. Our group has been studying a new analgesic substance called crotalphine (CRP), obtained from the venom of the South American rattlesnake *Crotalus durissus terrificus*. CRP induces potent and long-lasting antinociception mediated by activation of κ and δ opioid receptors, and due to its effectiveness in acute and chronic pain models, pre-clinical trials are being developed aiming to use this peptide as a new therapeutic analgesic. However, it is important to point out that the studies on the analgesic properties of CRP have been always developed in male animals. **Objectives:** The purpose of the present study was to evaluate the differences in pain sensation and in the antinociceptive response to CRP between male and female Wistar rats. These goals were assessed by the investigation of the differences between male and female rats to the hypernociceptive effect of carrageenan (Cg) and prostaglandin E_2 (PGE_2). The onset, peak, intensity and duration of the nociceptive responses were analyzed. In addition, the differences between male and female rats to the antinociceptive effect of CRP and morphine (positive control), regarding the onset, peak, intensity and duration of the antinociceptive response as well as the role of the female estrous cycle on pain threshold and CRP effectiveness were also analyzed. **Methods:** Hyperalgesia was determined using the rat paw pressure test. This test was applied before and at different times after the intraplantar injection of PGE_2 (50,100,150 ng/paw) or Cg (100, 200 μg/paw). For antinociception evaluation, CRP (5; 1; 0.2; 0.04; 0.008 μg/kg, p.o.) or morphine (5; 1; 0.2; 0.04; 0.008 mg/kg, s.c.) were administered immediately before or 2h after the hyperalgesic agent, respectively, and the test applied 3h after the hyperalgesic agent. To determine the influence of the estrous cycle, vaginal smears were taken daily, for four days, at 9 a.m., and observed with 10x and 40x light microscope objectives. The different phases of the estrous cycle are characterized as proestrus, estrus, diestrus and metestrus, with the presence of different cell types in each phase. **Results:** Female rats showed lower baseline pain threshold and responded to lower doses of PGE_2 (50 ng/paw) than did male rats. Female rats responded to a lower analgesic dose of CRP (0.04 μg/kg) than did males (0.2 μg/kg), with significantly greater antinociception than males (43% and 26%, respectively). Morphine produced significantly greater antinociception in males (91%) than female (44%) rats, and males responded to lower doses of morphine (0.008 mg/kg) in the PGE_2 model. No differences were observed for the analgesic effect of CRP in the different phases of the estrous cycle. **Discussion:** Sex differences could be observed between male and female rats in relation to pain threshold and antinociception. Besides that, CRP is able to induce antinociception in both male and female rats, but it is more effective in females. This could be a characteristic of the CRP or of κ and /or δ opioid receptor agonists.

Supported by Fapesp, COINFAR Pesquisa e Desenvolvimento Farmacêutica Ltda.

1.20 Analgesic effect induced by *Naja kaouthia and* an α-cobratoxin isolated from the same snake venom

Gutierrez VP[1], Chacur M[1], Zambelli VO[1], Mirtschin P[2], Cury Y[1]
[1]Laboratório de Fisiopatologia, Instituto Butantan, SP, Brazil; [2]Venom Supplies Pty Ltd, Tanunda, Austrália

Introduction: Snake toxins display many pharmacological actions such as myotoxic, neurotoxic, anti- or procoagulant, bactericidal, pro- or anti-inflammatory and antinociceptive activities. **Objectives**: The aim of the present study was to investigate the possible antinociceptive effect induced by *Naja kaouthia* (Nk) and by an α-cobratoxin (α-cobra) isolated from *Naja kaouthia* venom and to investigate the participation of peripheral opioid receptors in prostaglandin (PGE_2)-induced hyperalgesia. **Methods:** The rat paw pressure test, using PGE_2 as a nociceptive stimulus, was used to evaluate pain threshold. A force (in g) was applied before and 3 h after the injection of PGE_2 to the paw and the values that induced withdrawal of the paw were considered to represent the pain threshold. **Results:** Nk (40 μg) or α-cobra (20 μg) administered p.o. immediately before the intraplantar (i.pl.) injection of PGE_2 (100 ng/paw) decreased the hyperalgesia. Naloxone (1 μg/paw), a nonspecific opioid receptor antagonist, injected i.pl. simultaneously with PG, abolished the antinociceptive effect of the venom and α-cobratoxin. Nor-BNI (50 μg/paw), an antagonist of *kappa* opioid receptor, injected i.pl. simultaneously with PG, partially antagonized the antinociceptive effect of α-cobra. ICI 174.864 (10 μg/paw) ,an antagonist of *delta* opioid receptor, abolished this effect. CTOP (20 μg/paw) an antagonist of *mu* opioid receptor, did not modify the α-cobra effect. **Discussion:** These data suggest that *Naja kaouthia* and α-cobratoxin are able to induce an antinociceptive effect. *Kappa* and *delta* opioid receptors are involved in the antinociceptive effect of α-cobratoxin isolated from *Naja kaouthia* venom in PG-induced hyperalgesia.

Supported by Venom Supplies, Instituto Butantan

1.21 Leukocyte influx induced by *Bothrops insularis* snake venom (BiV) in mice

Souto P, Moreira V, Nascimento NG, Teixeira CFP
Laboratório de Farmacologia, Instituto Butantan, SP, Brazil

Introduction: *Bothrops insularis* snake is an endemic species restricted to the Queimada Grande Island, coast of São Paulo State, Brazil. The venom of this species is considered more toxic than that of other bothropic species, and the events involved in inflammatory reaction induced by BiV are still unknown. **Objective:** The aim of this study was to evaluate the leukocyte influx induced by *B. insularis* venom. **Methods:** Male Swiss mice (18-20 g) received intraperitoneal injection (i.p.) of BiV or sterile saline (control). Total and differential numbers of leukocytes were assessed in peritoneal washes harvested at selected periods of time after venom injection. Total leukocyte counts were determined in a Neubauer chamber after dilution in Turk solution (1:20 v/v) and differential cell counts performed on Rosenfeld stained smears. **Results:** BiV at doses of 0.01, 0.05 and 0.25 mg/kg induced a significant influx of leukocytes into the peritoneal cavities of animals from 3 h up to 24 h, with a peak at 6 h, persisting even at 48 h in comparison with controls. Differential cell counts showed a significant infiltration of polymorphonuclear cells (PMN) from 3 h up to 24 h, with a maximum at 3 h, with predominance of neutrophils, whereas the number of mononuclear cells increased at 48 h as compared with controls. In contrast, at a dose 0.25 mg/kg, BiV induced leukocyte influx into peritoneal cavities of mice from 6 h up to 48 h with a predominance of mononuclear cells at the same periods of time without infiltration of neutrophils. **Discussion:** These data are the first demonstration that BiV induces leukocyte influx into the site of its injection. At low doses, BiV stimulates infiltration of neutrophils in the early periods of inflammatory response and mononuclear leukocytes during the later phase. At high dose, this venom preferentially stimulates the infiltration of mononuclear leukocytes, an effect which has not been described for venom from other species of *Bothrops* snakes and which may reflect differences among species in venom components.

Supported by FAPESP, CNPq

1.22 Characterization of paw edema induced by *Bothrops erythromelas* snake venom in mice: pharmacological mediation and neutralization by *Bothrops* antivenom

Castro Jr NC, Araújo SD, Gonçalves LRC
Laboratório de Fisiopatologia, Instituto Butantan, SP, Brazil

Introduction: Venoms of *Bothrops* snakes can cause serious local reactions, such as intense inflammatory edema at the site of the bite, besides hemorrhage, local blisters and necrosis, which can cause great tissue loss. *Bothrops* venoms also usually cause systemic bleeding and hemostatic disorders. Treatment with antivenom is highly effective in eliminating the systemic effects caused by the venom, but not for the local reactions. Previous results indicate that edema induced by *Bothrops jararaca* could be better neutralized if anti-inflammatory drugs, such as dexamethasone, are applied in combination with serum therapy. **Objective:** The efficacy of this combination in treating the edema induced by other *Bothrops* venoms is unknown, and the aim of this study was to investigate this further. **Method:** The venom of *Bothrops erythromelas* (BeV) was chosen due to the importance of this snake in envenomation occurring in the northeast region of Brazil. First, a characterization of the edema induced by this venom was carried out and then its pharmacological mediation was studied, pre-treating groups of mice with promethazine, methysergide, dexamethasone, indomethacin, celecoxib, nordihydroguaiaretic acid (NDGA), L-nitroarginine methylester (L-NAME), pentoxifylline or captopril, in order to evaluate the participation of histamine, serotonin, eicosanoid derivates from COX, COX2 and LOX pathways, nitric oxide, TNF, and kinins, respectively, in the mediation of this edema. Treatments with antivenom and heparin were performed before and after BeV injection. Antivenom was also administered in combination with dexamethasone or heparin. Edema was evaluated 1, 2, 4, 6 and 24h after the venom injection by plethysmography. **Results:** BeV induces edema in a dose-effect manner. This edema reached a maximum 2h after venom injection, began to decrease at 4h and was residual at 24h. Treatments with dexamethasone, indomethacin and celecoxib significantly inhibited this edema. Minor but significant inhibitions were achieved with methysergide and NDGA treatments. *Bothrops* antivenom and heparin treatment also significantly inhibited the BeV induced edema. Conversely, treatment with promethazine, L-NAME and pentoxifylline did not alter the edema induced by BeV. Group treated with captopril presented an edema significant higher. *Bothrops* antivenom and dexamethasone, but not heparin, decreased the BeV-induced edema when administered up to 30 min after the venom. The combination of *Bothrops* antivenom and dexamethasone decreased the edema when applied up to 40 min after the venom. At this time, antivenom and dexamethasone administered alone were ineffective **Discussion**: Results suggest that this edema is mediated mainly by eicosanoids, particularly by those originating from cyclooxygenase pathways, but also by eicosanoids from the 5-lipoxygenase pathway, bradykinin and serotonin. The results also indicate that histamine, nitric oxide and TNF did not mediate this inflammatory event. The *Bothrops* antivenom contains antibodies that neutralize toxins that induce this inflammatory edema and, as already observed in *Bothrops jararaca*-induced edema, the combination of this antivenom with dexamethasone, but not with heparin, can be beneficial for treatment of inflammatory edema caused by *B. erythromelas* envenomation.

Supported by FAPESP, CNPq

1.23 Role of lipidic mediators in the local edematogenic reaction induced by *Bothrops moojeni* snake venom in mice

Nascimento NG, Olivo RA, Sampaio MC, Teixeira CFP
Laboratório de Farmacologia, Instituto Butantan, SP, Brazil

Introduction: *Bothrops moojeni* snake venom causes pronounced local tissue damage in humans and in experimental animals, characterized by hemorrhage, myonecrosis, pain and prominent edema. **Objectives:** In this study, the participation of lipid mediators in paw edema induced by *Bothrops moojeni* venom (BmV) was investigated. **Methods:** Male Swiss mice (18-20 g) were used. Paw edema was measured by plethysmography at selected time periods (15 min – 24 h) after injection of BmV (1 μg/paw) into a hind paw and saline into the contralateral paw (control). Groups of mice were treated with dexamethasone (1 mg/Kg, i.p.), a PLA2 inhibitor, 2 h before venom injection or with indomethacin (1 mg/Kg, i.v.), a non selective cyclooxygenase (COX) inhibitor, combined or not with ketotifen (5 mg/Kg, i.p.), a mast cell stabilizer, 30 min before venom injection or rofecoxib (6 mg/Kg, p.o.), a specific COX-2 inhibitor or zileuton (100 mg/Kg, p.o.), a 5-lipoxygenase inhibitor, 2 h before venom or respective vehicles (controls) in similar conditions. **Results:** BmV injection induced a marked paw edema from 15 min up to 6 h with a peak at 30 min after its injection. Paw volume returned to baseline levels at 24 h. Treatment of animals with dexamethasone decreased BmV-induced edema from 30 min up to 6 h. Indomethacin and refecoxib treatments significantly reduced paw edema induced by BmV from 15 min up to 3 h and from 1 h up to 3 h, respectively, whereas treatment with zileuton reduced paw edema from 30 min up to 1 h when compared with controls. Paw edema was abolished when indomethacin was combined with ketotifen. **Discussion:** Results indicate that arachidonic acid metabolites are important in the edematogenic reaction induced by BmV. COX-1 metabolites are important for the initial phase, whereas COX-2 metabolites are involved in the later phase of BmV-induced edema in mice. Moreover, the lipid mediators need to act in association with other mediators released by mast cells for complete development of edema caused by BmV.

Support by FAPESP, CAPES, SSPSP

1.24 Leukocyte influx induced by *Bothrops moojeni* snake venom (BmV) and participation of mast cells in this effect

Sampaio MC, Fernandes CM, Nascimento NG, Zamunér SF, Teixeira CFP
Laboratório de Farmacologia, Instituto Butantan, SP, Brazil

Introduction: Envenomation caused by *Bothrops moojeni* snake results in severe local effects in the victims with hemorrhage, myonecrosis and local inflammation. Many cells are important in inflammatory reaction such as the mast cells. These cells play a central role in the inflammatory reaction releasing an impressively broad array of inflammatory mediators. However, the mechanisms and the role of these cells in the inflammatory reaction induced by BmV are still unknown. **Objectives:** The aim of this study was to evaluate the leukocyte influx induced by *Bothrops moojeni* venom and participation of mast cells in this effect. **Methods:** Male Swiss mice (18-20 g) received intraperitoneal injection (i.p.) of BmV (0.125, 0.25 or 0.5 μg/g) or apyrogenic saline (control). Total and differential numbers of leukocytes were assessed in peritoneal washes harvested at selected periods of time after venom injection. Total leukocyte counts were determined in a Neubauer chamber after dilution in Turk solution (1:20 v/v) and the differential cells count performed in Hema^3 stained smears. Groups of mice were pretreated with ketotifen (5 mg/kg, i.p.), a mast cell stabilizer, 30 min before injection of BmV (0.25 μg/g) and total and differential leukocyte influx was evaluated at 6 h after venom injection. **Results:** BmV at all tested doses induced a significant influx of total leukocytes, neutrophils and macrophages into peritoneal cavities of mice at 6 h of injection. Maximal leukocyte peritoneal infiltration was obtained at the lowest dose. The time course of leukocyte influx induced by BmV at a dose of 0.25 μg/g showed a significant increase in total cell counts from 3 h up to 48 h, decreasing thereafter and reaching control values at 72 h. Maximal total leukocyte number occurred at 24 h. Differential cell counts showed a significant infiltration of polymorphonuclear (PMN) cells from 3 h up to 72 h, maximum at 6 h, with a predominance of neutrophils, whereas the number of mononuclear (MN) leukocytes increased significantly from 3 h up to 48 h with a peak at 24 h in comparison with controls. Ketotifen pretreatment caused a significant reduction in BmV-induced total and MN but not PMN leukocyte influx by 28% and 43%, respectively, at 6 h. **Discussion:** These results indicate that BmV is able to induce leukocyte influx into mouse peritoneal cavity, with predominance of neutrophils in the early periods and of macrophage in the later phase. Since ketotifen reduced BmV-induced leukocyte infiltration, mast cell mediators may be involved in this venom-induced effect. In conclusion, these data demonstrate that BmV induces a typical acute inflammatory event and that mast cells contribute to this effect.

Supported by FAPESP, CNPq, FUNDAP

1.25 Nitric oxide and Tumor Necrosis Factor α modulate the edematogenic reaction induced by *Bothrops moojeni* snake venom (BmV) in mice

Nascimento NG, Sampaio MC, Olivo RA, Souto P, Teixeira CFP
Laboratório de Farmacologia, Instituto Butantan, SP, Brazil

Introduction: Envenomation caused by *Bothrops moojeni* snake results in severe local effects both in humans and experimental animals with prominent edema, pain, hemorrhage and myonecrosis. **Objective**: The aim of this study was to evaluate the participation of NO and TNF-α in paw edema induced by BmV in mice. **Methods:** Male Swiss mice (18-20 g) were used. Paw edema was measured by plethysmography from 15 min up to 24 h after intraplantar injection of BmV (1 μg/paw) into a hind paw and saline (50 μL) into the contralateral paw (control). Groups of mice were treated with L-NAME (15 mg/Kg, i.v.), a non-specific inhibitor of nitric oxide synthase or aminoguanidine (50 mg/Kg, i.p.), a specific inhibitor of inducible nitric oxide synthase, 24 h and 30 min before venom or chlorpromazine (1.25 mg/Kg, i.p.), an inhibitor of TNF-α synthesis, 30 min before venom or respective vehicles (controls) in similar conditions. **Results**: BmV intraplantar injection induced a marked increase in mice paw volumes from 15 min up to 6 h with a peak at 30 min after its injection. The paw volume returned to baseline levels at 24 h. Treatment of animals with L-NAME caused a significant increase in BmV-induced paw edema from 3 h up to 6 h persisting even at 24 h, in comparison with control In addition, aminoguanidine treatment increased paw edema at 6 h, whereas chlorpromazine caused a significant decrease in BmV-induced edema from 15 min up to 6 h after its injection, when compared with control. **Discussion:** The results suggest that NO via both constitutive and inducible NO-synthases down-regulates paw edema induced by BmV and that TNF-α contributes to the development of this venom effect.

Supported by FAPESP, CAPES, SSPESP

1.26 The snake venom metalloproteinase BaP1 induces joint hypernociception through TNF α and PGE_2 dependent mechanisms

Fernandes CM[1], Rocha FA[2], Leite ACRM[2], Gutiérrez JM[3], Teixeira CFP[1].
[1]Laboratório de Farmacologia, Instituto Butantan, SP, Brazil; [2]Faculdade de Medicina, Universidade do Ceará, CE, Brazil; [3]Instituto Clodomiro Picado, Universidade da Costa Rica, Costa Rica

Introduction: Inflammatory arthropathies, including rheumatoid arthritis, affect about 1% of the general population and are among the leading causes of disability and premature mortality, especially in the elderly. Joint deformities are prominent in these patients and joint pain is the most frequent complaint and the main reason to seek medical attention. Matrix metalloproteinases (MMPs) are abundant in inflamed articular joints and have been implicated in cartilage destruction. However, the involvement of MMPs in the development of joint pain and inflammatory events is still unknown. **Objectives:** In this study, we examined the ability of BaP1, a metalloproteinase from *Bothrops asper* snake venom, with structural homology to MMPs, to induce: i) inflammatory reaction in rat articular joints, analyzing the vascular permeability (VP), leukocyte influx, TNF-α and PGE_2 release and histopathological changes; ii) joint hypernociception; iii) cyclooxygenase (COX) -1 and -2 expression by isolated macrophages and iv) the participation of TNF-α and PGE_2 in leukocyte influx and joint incapacitation. **Methods:** Male Wistar rats (200-250 g) received intra-articular (i.art.) injection of BaP1 (5 μg/joint) or bovine serum albumin (5 μg/joint-control). At selected time intervals, VP was quantified by extravasation of Evans blue and leukocyte influx by total and differential counts using a Neubauer chamber and stained smears (Hema3), respectively. Release of TNF-α was determined by cytotoxicity in the L929 cell line and PGE_2 by EIA. Histopathological changes were analyzed in synovial membrane sections stained with hematoxylin-eosin, the hypernociception determined by rat knee joint articular incapacitation test and COX expression by Western blotting. Groups of animals were pretreated with indomethacin (4 $mgKg^{-1}$ i.p.) or antiserum anti-rat TNF-α (50 μl i.art.) or their vehicles, 20 min before i.art. injection of BaP1, and leukocyte influx, joint incapacitation and PGE_2 release were measured. **Results:** The i.art. injection of BaP1 increased VP (1-6 h) and induced a marked influx of leukocytes into joints at all the periods studied (1-48h), maximal at 3 h, with predominance of polymorphonuclear cells. These effects were confirmed by histological analyses. BaP1 caused release of TNF-α and PGE_2 in synovial exudates, from 30 min up to 6 h. Further, BaP1 induced articular hypernociception from 1 up to 6 h. Pretreatment with indomethacin or antiserum anti-TNF-α significantly reduced hypernociception and leukocyte influx induced by BaP1. Antiserum anti-TNF-α did not affect BaP1-induced increase in PGE_2 levels in synovial exudates. Moreover, BaP1 induced expression of COX-2, but not COX-1, at all tested time intervals. **Discussion:** Our data are the first experimental evidence that a metalloproteinase has pro-nociceptive and pro-inflammatory activities in joints. These effects involve local release of PGE_2 and TNF-α. Both mediators contribute to BaP1-induced nociception and inflammation. BaP1-induced increase in PGE_2 is associated with increased COX-2 expression. Therefore, MMPs may be involved in joint hypernociception. Targeting MMPs may thus provide both symptomatic and structural benefit in inflammatory arthropathies.

Supported by FAPESP

1.27 Characterization of articular inflammation induced by Lys49 phospholipase A_2 (myotoxin II)

Dias RG[1], Sampaio SC[1], Picolo G[1], Sinhorini IL[2], Cunha FQ[3], Gutierrez JM[4], Lomonte B[4], Cury Y[1]

[1]Laboratório de Fisiopatologia, Instituto Butantan, SP, Brazil; [2]Laboratório de Patologia Experimental e Comparada, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo; [3]Departamento de Farmacologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, SP, Brazil; [4]Instituto Clodomiro Picado, Universidade da Costa Rica, Costa Rica

Introduction: Phospholipase A2 (PLA2) enzymes hydrolyze membrane phospholipids generating fatty acid and lysophospholipid. These events are thought to be the rate-limiting step in the generation of lipid pro-inflammatory mediators, the eicosanoids and also platelet-activating factor. For this reason, extracellular forms of PLA2 have been postulated to be a component of the inflammatory cascade in certain biological settings such as in the synovial fluid of patients with articular diseases. Furthermore, several data have shown that Lys49PLA2 (MII) isolated from *Bothops asper* venom is able to induce pain and inflammation. In this study, we proposed the use of venom PLA2 as a scientific tool for the standardization of a model of articular inflammation. **Objectives:** This work was aimed at developing a new model for studying articular inflammation, using MII isolated from *Bothops asper* venom. We characterized the alterations of vascular permeability (VP) and cell influx induced by the injection of MII in the rat knee joint. In addition, we evaluated the nociceptive effects caused by the toxin. **Methods:** To establish the dose of MII to be used in this study, we examined cell viability by the trypan blue exclusion method. The concentration that does not induce cell death was 10μg and this dose was chosen for the following studies. Alterations in VP were evaluated by assessing the extravasation of Evans blue (EB) into the rat articular cavity at several time intervals after injection of MII, BSA (positive control) or PBS (control). EB was injected 15 min before for assessment of extravasation. Cell influx was evaluated, in joint washes, at different times after injection of MII. Total and differential leukocyte counts were determined. Nociception was analyzed by an electronic pressure-meter, according to the method described by Guerrero et al[1], adapted for rats. Paraffin-embedded tissue sections were stained with HE for histopathological analysis. **Results:** The VP assays showed a significant increase in EB extravasation, 15 min after injection of MII when compared to rats that received BSA or PBS. The number of polymorphonuclear cells in the joint washes increased at 4 and 8 h after injection. The administration of MII into the tibio-tarsal joint of rats induced dorsal flexion-elicited hypernociception 4, 6 and 8 h after injection. Knee joint histological analysis showed significant inflammatory events, edema, cell influx and congestion, at 4 and 8 h after MII injection. Synovial edema and fibroplasia were also observed at 24 h after MII treatment. **Discussion:** The results indicate that MII is able to induce an articular inflammatory process, showing classic signs of acute inflammation. In addition, this toxin induces alterations that characterize nociceptive behavior. These data indicate that MII can induce acute arthritis, making it an important tool for the study of this disease.

(1) Guerrero, AT, Pharmacol Biochem Behav, vol 84, p. 244, 2006.

Supported by: Fapesp, CAPES

1.28 Lipid bodies formation induced by a secreted phospholipase A2 isolated from *Bothrops asper* snake venom

Leiguez Jr E[1], Zuliani JP[2], Lima SA[1], Cianciarullo AM[3], Gutiérrez JM[4], Teixeira CFP[1]

[1]Laboratório de Farmacologia, [3]Laboratório de Genética, Instituto Butantan, SP, Brazil; [2]Instituto de Pesquisas em Patologias Tropicais de Rondônia, RO, Brazil; [4]Instituto Clodomiro Picado, Universidade da Costa Rica**,** Costa Rica

Introduction: MT-III, a phospholipase A_2 isolated from *Bothrops asper* snake venom, induces important inflammatory events and stimulates many functions of isolated macrophages. In these cells, lipid rich cytoplasmic inclusions - lipid bodies (LB) - are increased under pathological conditions. **Objective:** The objective of this study was to evaluate the effect of MT-III on formation of LB in isolated macrophages. **Methods:** Macrophages were obtained from Swiss mouse peritoneal cavities 96 h after intraperitoneal injection of 3% thioglycolate. $2x10^5$ cells were incubated with MT-III (3.15; 6.3 and 12.6 μg/mL) or RPMI (control) for 1 h. Viability of cells and formation of LB were determined by reduction of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and 1% osmium tetroxide stain assays, respectively. LB were enumerated under phase contrast microscopy. **Results:** Incubation of macrophages with MT-III at concentrations of 3.15, 6.3 and 12.6 μg/mL did not affect cell viability but induced increases in the number of LB by 78%, 115% and 134%, respectively, in comparison with controls. **Discussion:** These data provide the first demonstration that a secretory phospholipase A_2 (MT-III) has the ability to induce LB in elicited macrophages. This effect depends on phospholipase A_2 concentration and may have implications in the inflammatory functions of macrophages.

Supported by FAPESP, CNPq

1.29 NFκB and cPLA$_2$ are involved in COX-2 expression and prostaglandins biosynthesis induced by two secretory PLA$_2$ (sPLA$_2$s) isolated from *Bothrops asper* venom (BaV)

Moreira V[1], Souto P[1], Gutiérrez JM[2], Teixeira CFP[1]
[1]Laboratório de Farmacologia, Instituto Butantan, SP, Brazil; [2]Instituto Clodomiro Picado, Universidade da Costa Rica, Costa Rica

Introduction: Four myotoxins with sPLA$_2$ structure were isolated from Bav. Two of them are the myotoxin-III (MT-III), with catalytic activity, and myotoxin-II (MT-II) which lacks enzymatic activity due to substitution of the amino acid Asp by Lys at position 49. Despite this difference, both display inflammatory effects. We previously showed that these sPLA$_{2s}$ promote the synthesis of PGD_2 and PGE_2, with distinct time courses and induce COX-2 expression in resident peritoneal macrophages. Considering that the transcription factor-kappa B (NF-κB) is important for induction of COX-2 protein and that intracellular PLA$_2$s such as cytosolic PLA$_2$ (cPLA$_2$) and Ca^{+2}-independent (iPLA$_2$) can cooperate with sPLA$_2$ in the biosynthesis of prostaglandins in leukocytes, we evaluated the involvement of these factors on MT-II- and III-induced expression of COX-2 and production of PGD_2 and PGE_2. **Methods:** Peritoneal resident macrophages were obtained from male Swiss mice and incubated with MT-II or III 6.5 μg/mL, for 4.5 or 6 h. The involvement of NF-κB in COX-2 expression was evaluated using the inhibitors TPCK (2.5 μM) and SN50 (50 μg/ ml). To study the participation of cPLA$_2$ and iPLA$_2$, the compounds $AACOCF_3$ (20 μM) and $PACOCF_3$ (4 μM) were used. COX-2 was determined by Western blotting and PGE_2 and PGD_2 by enzyme immune assay (EIA). **Results**: Preincubation of isolated macrophages with TPCK blocked COX-2 expression induced by MT-III and decreased by 50% the expression induced by MT-II ($p<0.05$ *vs* control). In addition, treatment of cells with compound SN50 significantly reduced COX-2 expression induced by MT-II and MT-III (50 and 70% average, respectively; $p<0.05$ *vs* control). Stimulation of macrophages with MT-II or MT-III increased the release of both PGD_2 (0.07±0.013; 0.6±0.1; 0.6±0.043 ng/mL for control, MT-II and MT-III, respectively; $p<0.05$) and PGE_2 (0.4±0.1; 12.8±0.8; 9.7±0.9 ng/mL for control, MT-II and MT-III, respectively; $p<0.05$). Inhibition of cPLA$_2$ by pretreating cells with compound $AACOCF_3$ markedly reduced PGD_2 and PGE_2 increments caused by both MT-II and MT-III ($p<0.05$) whereas inhibition of iPLA$_2$ by $PACOCF_3$ did not affect these parameters. However, these inhibitors did not change the expression of COX-2 induced by either myotoxin. **Discussion:** The data obtained indicate that NF-κB is critical in the induction of COX-2 by both MT-II and MT-III in macrophages and that cPLA$_2$ is important for their stimulatory effect on PGD_2 and PGE_2 synthesis, acting by a pathway different from COX-2 expression. In contrast, iPLA$_2$ does not participate in MT-induced up-regulation of PGs cascade. Since MT-II lacks enzyme activity, the phospholipase activity alone is not important for activation of this cascade. These findings show novel regulatory mechanisms for secretory PLA$_2$ in inflammatory cells.

Supported by FAPESP, CNPq.

1.30 Recombinant expression of the non-catalytic domains of the hemorrhagic metalloproteinase HF3, and their effect on cremaster muscle microcirculation

Menezes MC[1], Paes Leme AF[1], Bruni FM[1], Olivo RA[2], Teixeira CF[2], Della Casa MS[3], Lima C[1], Lopes-Ferreira M[1], Camargo ACM[1], Serrano SMT[1]
[1]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, [2]Laboratório de Farmacologia, [3]Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil

Introduction: HF3 is a metalloproteinase from *Bothrops jararaca* venom comprising catalytic, disintegrin-like and cysteine-rich domains. It causes potent local hemorrhage, degrades extracellular matrix proteins and inhibits platelet aggregation. Previously, we have studied the pro-inflammatory effect of this metalloproteinase and showed the ability of native HF3 and its recombinant disintegrin-like and cysteine-rich domains (DC/HF3) to activate macrophage phagocytosis through integrin αMβ2. Other studies with jararhagin-C, a protein comprising the disintegrin-like/cysteine-rich domains of jararhagin, showed that the non-catalytic domains of jararhagin are sufficient to locally activate the early events of an acute inflammatory response such as leukocyte rolling and pro-inflammatory cytokine release. **Objectives:** The aims of this study were to obtain the recombinant non-catalytic domains of HF3 and to compare their ability to affect the cremaster muscle microcirculation as evidenced by the activation of leukocyte rolling observed by intravital microscopy. **Methods:** Recombinant disintegrin-like+cysteine-rich (DC), disintegrin-like (D), and cysteine-rich (C) proteins were obtained in fusion with glutathione S- transferase (GST) in *E. coli* DH5α. The dynamic of alterations in the microcirculatory network was analyzed using intravital microscopy by transillumination of mouse cremaster muscle after topical application of samples (5 μM of protein dissolved in 20 μL of PBS). Control experiments were performed by applying 5 μM of GST under identical conditions. The local administration of each protein was monitored for 30 min and values averaged for three independent experiments. **Results:** The recombinant fusion proteins GST-DC, GST-D and GST-C were purified by affinity chromatography on glutathione Sepharose 4B and were shown to be essentially homogeneous by SDS–PAGE and by Western blot analysis using an anti-HF3 antibody. The recombinant proteins GST and GST-D did not affect the number of rolling leukocytes, which remained at the baseline level of ± 20 leukocytes per min. On the other hand, GST-DC and GST-C proteins increased the number of rolling leukocytes to 127 and 100 per min, respectively, in post-capillary venules in all periods analyzed. None of these proteins interfered with microvasculature hemodynamic parameters, such as vessel diameter, erythrocyte speed or blood flow rate. **Discussion:** The recombinant proteins were successfully expressed in *E. coli* as evidenced by SDS-PAGE and Western-blot analysis, and by N-terminal sequencing. Only the proteins containing the cysteine-rich domain (GST-DC and GST-C) were able to affect leukocyte rolling, confirming the role of this domain in the interaction of P-III metalloproteinases with their targets.

Support by CAT/CEPID-FAPESP

1.31 Contribution of proteinases and phospholipases of the *Bothrops jararaca* venom to the inflammatory reaction in mice

Zychar BC, Dale CS, Demarchi D, Gonçalves LRC
Laboratório de Fisiopatologia, Instituto Butantan, SP, Brazil

Introduction and Objective: Toxins classified as serine proteases, metalloproteases or phospholipases A_2, isolated from venom of *Bothrops* snakes can induce inflammatory reactions that contribute to the severity of local symptoms of the envenomation. Nevertheless, the real contribution of each one of these classes of toxins to the inflammatory effect induced by the whole venom is poorly understood, which motivated the present study. **Methods:** *Bothrops jararaca* venom (BjV) was treated with phenyl-methyl-sulfonyl-fluoride (PMSF), 1,10-phenanthroline (oPhe) or p-bromophenacyl bromide (pBPB), to inhibit those classes of enzymes. Inflammatory parameters induced by 1μg of treated venoms were evaluated and compared to that observed with non-treated venom. Edema was evaluated by plethysmography and hyperalgesia/allodynia was evaluated with von Frey filaments, at different times after venom injection into a mouse hind paw. Leukocyte-endothelial interactions (LEI) were evaluated 2 or 24 h after venom injection into the scrotum of mice, by intravital microscopy preparation of the cremaster muscle. In these experiments rolling, adhered or migrated leukocytes were counted in venules of the microcirculation. **Results:** BjV induced marked alterations of LEI. After injection of non-treated venom into the scrotum of mice, a significant decrease at 2 h, and a significant increase at 24 h of leukocyte rolling were observed, when compared to the saline-injected group. Besides, animals injected with non-treated BjV showed a marked increase in cellular adhesion at all time points studied, and also a large number of migrated cells at 24 h. The oPhe-treated venom induced significant differences in LEI. When compared with the group injected with non-treated venom, the group injected with the oPhe-treated venom displayed a significant decrease in adhered cells 2 h after venom injection, as well as in migration 2 and 24 h after venom injection. The results showed that edema and hyperalgesia/allodynia induced by oPHE-treated venom were markedly less intense than that observed with non-treated venom. Edema and LEI parameters induced by pBPB-treated venom were similar to that observed with non-treated venom, but hyperalgesia/allodynia was significantly lower. Inflammatory parameters induced by PMSF-treated venom did not differ from those induced by non-treated venom. **Discussion:** In conclusion, the results indicate a major importance of metalloproteases and suggest a secondary role of phospholipases and of serine proteases in inflammatory reactions induced by *Bothrops jararaca* venom.

Supported by FAPESP, CAPES

1.32 Phenol used as a preservative in *Bothrops* antivenom induces impairment of leukocyte endothelial interactions

Zychar BC[1], Castro Jr NC[1], Marcelino JR[2], Gonçalves LRC[1]
[1]Laboratório de Fisiopatologia, [2]Seção de Processamento de Plasmas Hiperimunes, Instituto Butantan, SP, Brazil

Introduction: Although serotherapy is effective in treating systemic symptoms caused by *Bothrops* envenomation, it is ineffective for treating local and inflammatory reactions occurring at the bite site. Such inefficacy has been attributed to the rapid onset of local manifestations, and to the fact that antivenom cannot reverse established lesions, nor neutralize endogenous mediators involved in the inflammatory process induced by *Bothrops* snake venoms. **Objective:** The effect of antivenom on abrogating cellular inflammatory events, e.g., on impairing the leukocyte-endothelial interactions induced by *Bothrops* snake venoms was investigated in the present study. **Method:** The *Bothrops jararaca* venom (1μg) was injected into the subcutaneous tissue of the scrotum of mice pre-treated or not with i.v. injection of *Bothrops* antivenom. The disturbances induced by the venom in leukocyte-endothelial interactions (LEI) in the microcirculation of the cremaster muscle in mice was evaluated 2 and 24 h after venom injection, using intravital microscopy. **Results:** Our findings showed that *Bothrops* antivenom *per se* induced changes in LEI, similar to those induced by *B. jararaca* venom, such as significant decrease in leukocyte rolling, and significant increase in leukocyte adhesion and migration, when compared with animals injected with vehicle. Moreover, mice injected intravenously with *Bothrops* antivenom developed adverse reactions, such as tremors and dyspnea. This effect was mostly due to the phenol used in *Bothrops* antivenom as a preservative, since phenol-free antivenom did not induce such adverse reactions or impair LEI but did abrogate venom-induced changes in LEI parameters. Pre-treatment of animals with promethazine did not prevent impairment of leukocyte adhesion and migration, nor did it block the development of tremor or dyspnea induced by the i.v. injection of either *Bothrops* antivenom or phenol, indicating the non-participation of histamine in these reactions. Nonetheless, the presence or absence of phenol in *Bothrops* antivenom did not modify the capacity of this antivenom to neutralize the hemorrhagic, coagulant or edema-forming activities of *B. jararaca* venom. **Discussion:** In conclusion, the present data suggest that *Bothrops* antivenom contains antibodies that can neutralize toxins that impair leukocyte-endothelial interactions, but phenol, which is used as preservative therein, can originate some adverse reactions observed following serotherapy.

Supported by FAPESP, CAPES

1.33 BnP1, a novel P I metalloproteinase from *Bothrops neuwiedi* venom: biological effects benchmarking relative to jararhagin, a PIII SVMP

Baldo C[1], Tanjoni I[1], León IR[2], Batista IFC[3], Della Casa MS[1], Clissa PB[1], Weinlich R[4], Lopes-Ferreira M[5], Lebrun I[3], Amarante Mendes GP[4], Rodrigues VM[6], Perales J[2], Valente RH[2], Moura da Silva AM[1]

[1]Laboratório de Imunopatologia, [3]Laboratório de Bioquímica e Biofísica, [5]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, Instituto Butantan, SP, Brazil; [4]Laboratório de Biologia Celular e Molecular, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil; [6]Laboratório de Química de Proteínas, Universidade Federal de Uberlândia, MG, Brazil; [2]Laboratório de Toxinologia, FIOCRUZ, RJ, Brazil

Introduction: Snake venom metalloproteinases (SVMPs) have been extensively studied and their effects associated with the local bleeding observed in human accidents by viper snakes. Representatives of P-I and P-III classes of SVMPs similarly hydrolyze extracellular matrix proteins or coagulation factors, while only P-III SVMPs induce significant hemorrhage in experimental models. **Objectives:** In this work, our aim was to elucidate the mechanisms involved in venom-induced hemorrhage by comparing the effects of BnP1 and jararhagin (P-I and P-III SVMPs, respectively) on coagulation proteins and cultures of muscle and endothelial cells. **Methods:** BnP1 was isolated from *B. neuwiedi* venom by size exclusion and anion-exchange chromatography. The partial amino acid sequence of BnP1 was carried out by mass spectrometry and Edman degradation. Hemorrhagic activities of toxins were evaluated by *i.d.* injection of toxins on the dorsum of mice as well as by intravital microscopy. The myotoxic activity was assayed by creatine-kinase activity. The proteolysis of fibrinogen and fibrin was evaluated by SDS-PAGE and fibrin-agarose plates, respectively. We also compared the effects of BnP1 and jararhagin on HUVECs and C2C12 cells by viability and cell adhesion (MTT method), necrosis (LDH quantification), and apoptosis analyses (FACS). **Results and Discussion:** BnP1 showed apparent molecular mass of approximately 23.6 kDa and sequence similarity with other members of SVMPs, which allowed its classification as a group P-I SVMP. The comparison of local effects induced by SVMPs showed that BnP1 was devoid of both myotoxic and hemorrhagic activities and jararhagin showed only hemorrhagic activity. BnP1 and jararhagin were able to hydrolyze fibrinogen and fibrin, although the latter displayed higher activity in both systems. Using HUVEC primary cultures, we observed that BnP1 induced cell detachment and a decrease in the number of viable endothelial cells at levels comparable to those observed by treatment with jararhagin. Moreover, both BnP1 and jararhagin induced apoptosis in HUVECs, while only a small increase in LDH supernatant levels was observed after treatment with jararhagin, suggesting that the major mechanism involved in endothelial cell death is apoptosis. Jararhagin and BnP1 induced little effects on C2C12 muscle cell cultures, characterized by a partial detachment 24 h after treatment and a mild necrotic effect as evidenced by a small increase in supernatant LDH levels. Taken together, our data show that P-I and P-III SVMPs have comparable effects except for the hemorrhagic activity, suggesting that hydrolysis of coagulation factors or damage to endothelial cells is not sufficient for induction of local bleeding.

Supported by FAPESP, IOC-FIOCRUZ, CNPq

1.34 Inflammatory response induced by BnSP 7, a myotoxin isolated from *Bothrops neuwiedi pauloensis* snake venom

Oliveira CF[1], Lopes DS[2], Rodrigues VM[1], Moura da Silva AM[2], Clissa PB[2]
[1]Laboratório de Química de Proteínas e Produtos Naturais, Universidade Federal de Uberlândia, MG, Brazil; [2]Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil

Introduction: Snakebite envenomation constitutes an important public health hazard in Latin America. Most accidents are inflicted by species of the genus *Bothrops*. In addition to systemic alterations, these poisonings are characterized by prominent local tissue damage due to myonecrosis, hemorrhage and edema. Acute muscle damage induced by these venoms is mainly due to myotoxic phospholipases A_2 (PLA_2). **Objectives:** This work reports on the local inflammation induced by BnSP-7, a myotoxin isolated from *Bothrops neuwiedi pauloensis* snake venom, BnSP-7 modified by 4-bromophenacyl bromide - BPB (BnSP-7-BPB) and crude venom from that snake, measured by cytokine release. **Methods:** Inflammation was studied by determination of the levels of the cytokines IL-1β, IL-6, KC and TNF-α released in muscle and MPAC (murine peritoneal adherent cells). At 2, 4, 6, 8 and 24 h after injection of 25 μg of toxins and crude venom into mouse gastrocnemius muscle, the muscle was extracted, homogenized in PBS and centrifuged. The supernatant was used to quantify the cytokines. MPACs were obtained from mouse peritoneum pre-injected with thioglycolate. The cells were treated with 5μg of toxins and crude venom and the supernatant was collected at the same time intervals as described before. PBS was used as control. Quantification of cytokines was performed by ELISA. **Results:** BnSP-7, BnSP-7-BPB and crude venom increased the release of IL-1β, IL-6 and KC in both experimental models. The cytokines released at the highest levels by muscle and MPAC were IL-6, reaching a peak at 4 h after toxin injection, and KC, reaching a peak at 6 h. The release of TNF-α was not significant in both models. The results showed also that the modification of BnSP-7 with BPB decreased the release of KC, IL-6 and IL-1 β in muscle when compared to native BnSP-7. **Discussion:** We conclude that both experimental models used in this work are effective in showing the profile of pro-inflammatory cytokines released for all toxins tested. Since BnSP-7 is a Lys49 PLA_2 and lacks enzymatic activity, the decrease in the release of some cytokines induced by BnSP-7-BPB may be caused by conformational changes in regions of the molecule different from the catalytic site.

Supported by CNPq, FAPESP

1.35 Neuwiedase effect on release of pro-inflammatory cytokines

Lopes DS[1], Oliveira CF[2], Rodrigues VM[2], Moura da Silva AM[1], Clissa PB[1]
[1]Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil; [2]Laboratório de Química de Proteínas e Produtos Naturais, Universidade Federal de Uberlândia, MG, Brazil

Introduction: Neuwiedase is a fibrinogenolytic nonhemorrhagic PI- SVMP from *Bothrops neuwiedi pauloensis* venom, which causes severe local damage characterized by myonecrosis, edema and an acute inflammatory reaction. **Objectives**: In the present study, we investigated the effect of neuwiedase on cytokine release in two different experimental models. Our *"in vitro*" model was carried out in murine peritoneal adherent cells (MPAC) and the "*in vivo"* model was carried out in mouse gastrocnemius muscle. **Methods**: To evaluate the direct effect of neuwiedase on the cell culture, MPACs (obtained from BALB-c mice pre-injected with thyoglycolate) were incubated with crude venom (5μg/ml), native neuwiedase or EDTA-inactivated neuwiedase (10μg/ml) and EDTA or PBS as controls. The levels of pro-inflammatory cytokines IL-6, IL-1β, TNF-α and KC in the culture supernatants were determined by ELISA at 2, 4, 6, 8 and 24 h after incubation. To evaluate the local effects, BALB-c mice were injected in the gastrocnemius muscle with 10 μg/animal of the same toxins cited before and EDTA or PBS as controls. The levels of pro-inflammatory cytokines were determined in supernatant of muscle homogenates, collected 2, 4, 6, 8 and 24 h after intra-muscular injection of samples. **Results**: Our "*in vitro*" experiments showed that neuwiedase induced a significant release of KC and TNF-α by MPACs, reaching a peak at 8 and 2 h after stimulation, respectively, while neuwiedase/EDTA induced approximately 50% less compared with neuwiedase. The release of IL-1β and IL-6 was not detected in the cell culture supernatant. The injection of neuwiedase into the gastrocnemius muscle induced a local increase of IL-6, IL-1β and KC, reaching a peak in production at 2 h after injection. Surprisingly, neuwiedase treated with EDTA induced higher levels of TNF-α when compared with neuwiedase or EDTA separately. This profile was observed for the other cytokines as well. **Discussion**: Our results show that neuwiedase is able to induce the release of pro-inflammatory cytokines by MPAC and that this effect is not dependent on catalytic activity. The mechanism responsible for the increase of cytokines in the gastrocnemius muscle induced by EDTA-inactivated neuwiedase needs to be further investigated.

Supported by CNPq, FAPESP

1.36 The coagulant effect of different snake venoms on purified *Bothrops jararaca* (*Bj*) fibrinogen

Vieira CO[1,2], Sano Martins IS[1,2], Tanaka Azevedo AM[1]
[1]Laboratório de Fisiopatologia, Instituto Butantan; [2]Departamento de Fisiologia, Instituto de Biociências, Universidade de São Paulo, SP, Brazil

Introduction: Most Brazilian venomous snakes have in their venom a thrombin-like activity, i.e. an enzyme similar to thrombin, which cleaves fibrinogen and produces fibrinopeptide A. Proteinase inhibitors with anticoagulant and antihemorrhagic activities have been described in the plasma of several species of snakes and mammals. In addition, a thrombin inhibitor has also been identified in *Bothrops jararaca* snake plasma. However, taking into consideration the little understanding about the coagulation factors in snakes, it is difficult to evaluate the coagulation system in these animals. **Objectives:** The aim of this work was to isolate *Bj* fibrinogen, a soluble plasma glycoprotein that is converted by thrombin into insoluble fibrin monomers, as well as to evaluate the effect of some snake venoms on it. **Methods:** *Bj* fibrinogen was obtained from plasma by barium chloride adsorption, ammonium sulfate precipitation and gel filtration chromatography. **Results and Discussion:** The molecular masses of *Bj* fibrinogen chains were 71, 60 and 55 for Aα, Bβ and γ, respectively, by SDS-PAGE. Clotting assays were carried out using Bj and human fibrinogen as substrates to venoms of *Crotalus durissus terrificus*, *Bothrops jararaca* and *Lachesis muta rhombeata*; they could not clot *Bj* fibrinogen while they clotted human fibrinogen (minimum coagulant dose: 11.8 μg mL^{-1}, 4.46 μg mL^{-1} and 2.5 μg mL^{-1}, respectively). These results indicate that *Bj* fibrinogen is adapted to protect these animals against the self-clotting activity of thrombin-like enzymes present in its venom.

Supported by FAPESP, CNPq.

1.37 Low molecular weight fraction from *Bothrops jararaca* snake venom promote the disruption of mouse seminiferous epithelium through the nitric oxide production pathway

Pannocchia MA[1], Gilio JM[1], Portaro FCV[2], Fernandes BL[1], Camargo ACM[1], Silva CA[1]

[1]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID; [2]Laboratório de Inunoquímica, Instituto Butantan, SP, Brazil

Introduction: A molecular mechanism of action for NO in spermatogenesis has been proposed in recent studies showing that NO likely regulates tight junction (TJ) dynamics in the testis via the cGMP/protein kinase G and adherens junctions (AJ), in part, via the NOS signaling pathway. These studies have clearly illustrated the interesting role of NOS and NO in Sertoli-germ cell AJ restructuring, particularly in the way it regulates the cell adhesion of the CDH2/CATNB/actin protein complex in the seminiferous epithelium. Recently, we have shown that the low molecular weight fraction (LMWF) from *Bothrops jararaca* venom causes a significant disruption of the seminiferous epithelium and inhibition of spermiogenesis, transformation of spermatid into spermatozoon, in mice. In this work, we investigated if the disruption of seminiferous epithelium could be explained by the action of NO. **Objectives:** The aim of this study was to determine the level of NO and expression of NOS in mouse testis treated with L-NAME, nitroprusside and LMWF. **Methodology:** Three male Swiss mice (35g) were treated with L-NAME (L group), sodium nitroprusside (N group) or LMWF (5μg) in the left testis and vehicle in the right testis by intratesticular injection. On day 7, animals were killed and their testes were homogenized on ice with a polytron PT MR 3000 homogenizer (Kinematic AG, Littau) in 50 mM Tris-HCl buffer, containing 0.1 mM EDTA, 0.1 mM EGTA, 2mM 2-mercaptoethanol, and 1mM phenylmethylsulfonyl fluoride, pH 7.4. Subsequently, 1% TCAm (4°C) was added and the samples were centrifuged for 7 min at 13,000 *g*. The nitric oxide levels were determined by nitrate and nitrite accumulations in the supernatant (30 μL) of total testis protein extract in a NO analyzer (NOA^{TM280}; Sievers Inc.). eNOS and iNOS expression were analyzed by Western blotting of total testis protein extract using anti-eNOS and anti-iNOS (Zymed®)**. Results:** Group L, showed severe degenerative changes of the seminiferous epithelium of the left testis, such as increased epithelium height and decreased lumen diameter of tubules. On the other hand, in group N, there were focal areas showing germinative cells distributed in the lumen, and a decrease in epithelium height. Morphological parameters of the LMWF group were significantly similar to group N. The levels of NO of left testis in the N and LMWF groups (326μM; 119μM) were higher than those in the L group (2μM) ($P<0.01$). On the other hand, the levels of NO of right testes in the three groups were not significantly different ($P>0.05$). To confirm this hypothesis, eNOS and iNOS expression are being analyzed by Western blotting. **Discussion:** Changes in NO levels in the testes of mice treated in the L-NAME and sodium nitroprusside may be the cause for testis damage and disturbances of spermatogenesis as described in the literature. Moreover, we suggest that there are some compounds in the LMWF that increase NO levels, promoting a disruption of the seminiferous epithelium of mice, opening new perspectives for the medical development of a male contraceptive.

Support by CAT/CEPID-FAPESP, UNIEMP, CNPq

1.38 Two bradykinin-potentiating peptides from *Bothrops jararaca* venom, BPP11e and BPP 11eAP, show different effects in spermatogenesis mice

Gilio JM[1], Portaro FCV[2], Rioli V[1], Fernandes BL[1], Borella MI[3], Camargo ACM[1], Silva CA[1]

[1]Laboratório Especial de Toxinologia Aplicada- CAT/CEPID; [2]Laboratório de Inunoquímica, Instituto Butantan, SP, Brazil; [3]Laboratório de Endocrinologia de Peixes, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil

Introduction: The low molecular weight fraction (LMWF) of snake venom comprises a series of bioactive peptides, such as bradykinin potentiating peptides (BPPs). One of its mechanisms of action is related to inhibition of the somatic angiotensin converting cnzyme (sACE). BPPs 11e and 11e-AP from *B. jararaca* venom inhibit sACE, oligopeptidases 24.15 and 24.16, with K_i in the μM range. Recently, we have shown that LMWF from *Bothrops jararaca* causes inhibition of the process of spermiogenesis, transformation of spermatid into spermatozoon, in mouse spermatogenesis. These data indicated that such effects could occur via the interaction of BPPs with the testicular ACE (tACE), an enzyme expressed in germ cells exclusively during spermatogenesis. Studies have shown the involvement of tACE in male fertility, in fertilization as well as in spermatogenesis. **Objectives:** This study was conducted to compare the effect of BPP-11e and BPP-11eAP on the seminiferous epithelium of mice by intratesticular injection (i.t.). **Methods:** Male Swiss mice (35g) were treated with BPP-11e or BPP11-eAP (5μg) in the left testis and vehicle in the right testis, and after 7 days the animals were killed. Testes were removed and analyzed by Mallory trichromic staining or immunohistochemistry using streptavidin-biotinylated-peroxidase complex (Dako Cytomation) in order to study the expression and distribution of claudin-1, one of the tight junction proteins of the seminiferous epithelium. Morphological and morphometric parameters were analyzed in approx. 50 sections using a Zeiss Axioskop 2 photomicroscope. All data were presented as means ± SEM, and the criterion for statistical significance was set at $P<0.05$ (GraphPad Prism 4.0, GraphPad Software, Incorporation). **Results:** Seminiferous tubule morphology observed in animals treated with BPP-11eAP showed an intense induced disruption of the epithelium, presence of atypical multinucleated cells in the lumen, and degenerated germ cell in the adluminal compartment, when compared with morphology of the right testis. Morphometric parameters showed significant reduction ($P<0.001$) of round spermatids (7.75±1.50), spermatocytes (zygotene, 7.25±1.50; pachytene, 5.75±0.86) when compared to the right testis (29.5 ± 0.5, 15.2 ± 2.5 and 24.5±3.1, respectively). Interestingly, no morphological or morphometric alterations were observed in animals treated with BPP-11e. Immunohistochemistry studies showed that the distribution of claudin-1 was changed when animals were treated with BPP-11eAP, but not with BPP-11e or in control. **Discussion:** These data suggest that the effects cannot be attributed to the interaction of BPPs with the tACE or oligopeptidases (24.15 or 24.16), because the alterations in the spermatogenesis were observed only in animals treated with BPP-11eAP. Thus, these results open perspectives for understanding the effects BPP-11eAP have on spermatogenesis.

Supported by CAT-CEPID-FAPESP, UNIEMP, CNPq

1.39 Identification of the molecular targets of bradykinin potentiating peptide 10c in the central nervous system

Oliveira EF, Guerreiro JR, Gomes CL, Fernandes BL, Camargo ACM, Silva CA
Laboratório Especial de Toxinologia Aplicada- CAT/CEPID, Instituto Butantan, SP, Brazil

Introduction: The bradykinin-potentiating peptides (BPPs), produced in the venom glands of a few snakes, were the first natural somatic angiotensin-converting enzyme (sACE) inhibitors to be described. Vane suggested that the inhibition of the sACE causes the observed antihypertensive activity of the BPPs, which are found in the venom of *Bothrops jararaca* (*Bj*). The *in vivo* experiments had shown that the *Bj*-BPPs blocked bradykinin degradation and inhibited the conversion of angiotensin I (Ang I) to angiotensin II. However, it has been suggested that some *Bj*-BPPs may act by an ACE-independent mechanism. Biodistribution studies in mice showed that the BPP-10c (<ENWPHPQIPP) accumulated in a few organs, both in the absence and presence of a 1000-fold excess of captopril (specific inhibitor of ACE), suggesting the existence of other molecular targets. In addition, biochemical and pharmacological features of the brain *Bj*-BPPs, such as their presence within the neuroendocrine regulator C-type natriuretic peptide (CNP) precursor, and their expression in regions of the snake brain correlated to neuroendocrine functions, have suggested that these peptides belong to a novel class of endogenous vasoactive peptides. **Objectives:** This study was conducted to identify new possible targets for the BPP-10c in the central nervous system (CNS) of mice using an affinity chromatography strategy. **Methods:** Approximately 1 mg BPP-10c was dissolved in 1 ml of coupling buffer ($NaHCO_3$0.2 M, NaCl0.5 M; pH 8.3), and the solution was coupled to 1 ml of HiTrap NHS-activated HP resin according to the instruction manual of Pharmacia. Cytosolic and membrane fractions proteins were obtained from mouse brain and binding studies were performed with 20 mM Tris-HCl (pH 8.0) containing 5 mM DTT. The fraction eluted from affinity chromatography was analyzed by SDS-PAGE, and the proteins were identified by MALDI-TOF/MS using α-cyano-4-hydroxycinnamic acid (Sigma) as matrix on an Ettan Maldi-Tof/Pro instrument (Amersham Biosciences). **Results:** SDS-PAGE analysis of the eluted membrane fraction demonstrated five proteins (86.1, 62.3 58.4, 47.8 and 36.1 kDa) with affinity to BPP-10c. Mass spectrometry analysis of proteins showed: 1) aconitase 2, mitochondrial; 2) pyruvate kinase 3; 3) glucose phosphate isomerase 1; 4) Aspatate aminotransferase precursor; and 5) glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **Discussion:** Recently, we observed a sustained antihypertensive activity of *Bj*-BPPs in spontaneously hypertensive rats (SHRs) which was not related to the inhibition of sACE in vitro. In addition, the bradykinin potentiation or blockade of the pressor effect of angiotensin I *in vivo* was not observed. Biochemical studies of proteins showing affinity to BPP-10c could be new targets to explain antihypertensive activity of *Bj*- BPPs.

Supported by FAPESP

1.40 Platelet aggregation inhibition by snake venom metalloproteases

Ventura JS[1], Ramos OHP[1], Moura da Silva AM[2], Chudzinski Tavassi AM[1]
[1]Laboratório de Bioquímica e Biofísica, [2]Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil

Introduction: Jararhagin and berythractivase are two P-III snake venom metalloproteases (SVMPs) that exhibit high structural similarity (79.40% of similar residues) but divergent biological effects. In contrast to berythractivase, jararhagin is a highly hemorrhagic enzyme that does not activate prothrombin. **Objectives:** Our aim in the present study was to compare these homologous proteins concerning their effects on platelet aggregation and their interactions with platelet receptors. **Methods:** Due to berythractivase's procoagulant activity, all experiments were conducted using washed platelets ($300x10^9$/L). For platelet aggregation assays, collagen (2μg/ml) or thrombin (1 U/ml) were used as inducers after pre-incubation (37°C, 10 min) with jararhagin (2μM) or berythractivase (5μM). In platelet receptor recognition assays, jararhagin and berythractivase (5μM) were also pre-incubated with EDTA (5mM) to probe the requirement of metals for their interactions with GPIa (α2 integrin), GPIIa (β1 integrin) and GPIbα, following addition of FITC-labeled antibodies and flow cytometry analysis. **Results:** Jararhagin and berytrhactivase were able to significantly inhibit platelet aggregation induced by collagen (IC_{50} = 0.3 μM and IC_{50} = 1.25 μM, respectively), while thrombin-induced platelet aggregation was only slightly inhibited by them (31% and 13%, respectively, using 5μM of the enzymes). They were also able to block GPIa and GPIbα but not GPIIa, recognition by monoclonal antibodies. Pre-incubation of the SVMPs with EDTA abolished their binding to GPIbα but did not influence the GPIa binding. **Discussion:** Since metal removal of P-III SVMPs implies not only the loss of enzymatic activity but also conformational changes in metal coordinated regions, we propose that, in contrast to GPIa recognition, the GPIbα interactions with the enzymes involve proteolysis or are related to conformational regions associated with metal coordination. Structural bioinformatics predictions point to interactions of GPIbα with all 3 domains of both SVMPs.

Support by FAPESP, FINEP

1.41 Effects of *Lonomia obliqua* bristle extract on blood platelets

Carrijo-Carvalho LC, Ventura JS, Oliva DP, Seibert CS, Chudzinski-Tavassi AM
Laboratório de Bioquímica e Biofísica, Instituto Butantan, SP, Brazil

Introduction: The *Lonomia obliqua* bristle extract has procoagulant proteins that *in vivo* cause a consumption coagulopathy resulting in depletion of coagulation factors and bleeding. Patients envenomed by accidental contact with the *L. obliqua* caterpillar present increased coagulation time and decreased fibrinogen levels. In addition, there are reports of a moderate thrombocytopenia. However, the effects of *L. obliqua* bristle extract on platelet function are not known. **Objectives:** The aim of the study was to determine the effects of *Lonomia obliqua* crude bristle extract (LOCBE) on platelet aggregation *in vitro* and *in vivo* and on hematological parameters in an animal model. **Methods:** Male BALB/c mice weighing 18-22 g (8 per group) were given 15 μg of LOCBE intraperitoneally in the treated group or the vehicle phosphate-buffered saline (PBS) in the control group. After 2 h, the animals were anesthetized and blood was collected by cardiac puncture. Platelet function was evaluated in treated and control animals by a whole blood platelet aggregation assay (Aggrolink, Chronolog) using collagen as the agonist. The hematological parameters evaluated were: prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen level and complete hemogram. The *in vitro* assays were carried out with human whole blood from healthy donors incubated for 15 min with LOCBE at various concentrations (2 – 20 μg/mL) prior to collagen addition. Platelet aggregation was monitored after addition of LOCBE during the incubation time until 15 min after induction by the agonist. **Results:** *In vitro*, LOCBE did not cause alterations in platelet function, either by stimulating or inhibiting platelet aggregation after addition of collagen. Mice treated with LOCBE showed a decrease in platelet aggregation (32% ± 7.4%) in comparison with the control group (83.8% ± 8.3%). The coagulation time was increased in the treated group (PT = 71.5 ± 26.2 s; APTT = 95.7 ± 31.1 s) in comparison with the control group (PT = 16.8 ± 1.3 s; APTT = 29.0 ± 4.5 s) and fibrinogen was depleted (control group = 347.7 ± 79.6 mg/dL; treated group = undetectable). Parameters in hemogram analysis were similar between treated and control group, except for platelet count which was reduced and leukocyte count which was increased in treated mice from 3.5 ± 1.8 (10^9/L) to 6.9 ± 0.7 (10^9/L), indicating that LOCBE has a pro-inflammatory action. **Discussion:** All these results taken together indicate that LOCBE have no direct effects on platelets, but it can affect platelet function by an indirect action in the organism. The mechanisms responsible for the alterations seen in platelet aggregation have to be investigated. They can be related, at least partially, to a reduction in platelet count and the consumption coagulopathy induced by the venom.

Supported by Fapesp, CNPq

1.42 Exploring the variability of *Bothrops jararaca* venom by proteomic analysis

Zelanis A[1,2], Gois C[1], Menezes MC[1], Camargo ACM[1], Furtado MFD[3], Ho PL[4], Serrano SMT[1]

[1]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, [3]Laboratório de Herpetologia, [4]Centro de Biotecnologia, Instituto Butantan, SP, Brazil; [2]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, SP, Brazil

Introduction: The venom of *Bothrops jararaca* is one of the most explored in Brazil due to the large geographical distribution of this snake and the number of envenomation cases. Although a number of toxins have been isolated from this species the use of powerful tools such as proteomic and transcriptomic techniques have improved the understanding of this venom as a whole. Snake venom proteome variability has long beenappreciated by investigators and it is a well-documented phenomenon. Previous studies have demonstrated individual, sex-based and ontogenetic variations in *Bothrops* venoms. **Objectives:** The aim of this study was to assess the variability of *B. jararaca* venom concerning two stages of the snake's life and gender. **Methods:** A total of 694 two-week-old newborns and 110 adults (49 males and 61 females, older than 3 years) from São Paulo state were milked and the venom lyophilized. Two-dimensional electrophoresis (2D PAGE) was carried out using a linear pH (3-10) gradient (7 and/or 24 cm IPG strips). Gel filtration chromatography was developed on Superose 12 HR 10/30 column, previously equilibrated with ammonium bicarbonate buffer. Coagulant activity was measured on citrated human plasma. 2D PAGE zymography was developed using acrylamide gels co-polymerized with gelatin. **Results and Discussion:** 2D PAGE revealed clear distinct profiles between newborn and adult and between male and female venoms. Image analysis of 2D gels showed a higher abundance of acidic proteins of high molecular mass in newborn and in male venoms. In addition, newborn venom showed a 13 times higher coagulant activity on plasma than adult venom, while no significant difference was detected between male and female venoms. 2D gelatin zymography showed high proteolytic activity only in adult venoms. Gel filtration chromatograms showed distinct profiles between newborn and adult venoms, but the highest specific coagulant activity was detected in the protein peak containing high molecular mass, acidic proteins of both venoms. Serine proteinase and metalloproteinase subproteomes were assessed by 2D-immunostaining and revealed slight differences between adult and newborn venoms, and between male and female venoms. Taken together, these results suggest the presence of different sets of proteins in the venom at two distinct phases in the life of *B. jararaca* and the differential expression of proteins between male and female venoms.

Supported by CAT/CEPID-FAPESP

1.43. Molecular cloning, expression and function of a new member of a gene family of Sphingomyelinases from *Loxosceles laeta* venom glands

Santi-Ferrara GI[1], Fernandes-Pedrosa MF[1,2], Junqueira de Azevedo ILM[3], Gonçalves-de-Andrade RM[1], Portaro FCV[1], Manzoni-de-Almeida D[1], van den Berg CW[4], Ho PL[3], Tambourgi DV[1]

[1]Laboratório de Imunoquímica, [3]Centro de Biotecnologia, Instituto Butantan, SP, Brazil; [2]Departamento de Farmácia, Universidade Federal do Rio Grande do Norte, RN, Brazil; [4]Departamento de Farmacologia, Terapêutica e Toxicologia, University of Wales, College of Medicine, Cardiff, UK

Introduction: The bite of spiders of the genus *Loxosceles* can induce a variety of biological effects, including dermonecrosis and complement-dependent hemolysis. Due to the limited understanding of the venom's mechanism of action, effective treatment is still not available. Several studies have indicated that sphingomyelinase D (SMase D) present in the venom of *Loxosceles* spiders is the main component responsible for the local and systemic effects observed in loxoscelism. Using "Expressed Sequencing Tag" strategy of random clones from *L. laeta* venom gland cDNA library we have identified clones containing inserts coding for proteins with significant similarity with the sphingomyelinases D. **Objectives**: To analyze the structural elements important for SMase D activity, the cDNA insert from one of these clones, H10, was expressed and the recombinant protein compared with the previously expressed and characterized SMase I from *L. laeta*, in terms of its biological, biochemical and structural properties. **Methods:** The cDNA insert from clone H10 was further subcloned in a pRSET-B bacterial expression system and the protein expressed as a 35-kDa-fusion protein containing a 6xHis-tag at its N-terminus. **Results and Discussion:** The H10 recombinant *L. laeta* spider venom protein, although sharing only 40% homology with the SMase I, was endowed with all biological properties ascribed to the whole *L. laeta* venom and the recombinant SMase I from *Loxosceles*, including dermonecrotic and complement-dependent hemolytic activities and ability of hydrolyzing sphingomyelin. Considering its activity, the recombinant new toxin expressed from clone H10 was named SMase II from *L. laeta*.

Supported by FAPESP, CNPq

1.44 Neutralization potential of a horse serum, produced against Sphingomyelinases D, on the toxic effects of *Loxosceles* spider venoms

Manzoni de Almeida D[1], Fernandes-Pedrosa MF[1,2], Gonçalves-de-Andrade RM[1], Marcelino JR[3], Ho PL[4], Junqueira de Azevedo ILM[4], Higashi HG[4], Tambourgi DV[1]

[1]Laboratório de Imunoquímica, [3]Seção de Processamento de Plasmas Hiperimunes, [4]Centro de Biotecnologia, Divisão Bioindustrial, Instituto Butantan, SP, Brazil; [2]Departamento de Farmácia, Universidade Federal do Rio Grande do Norte, RN, Brazil

Introduction: Envenomation by arachnids of the genus *Loxosceles* (brown spider), endemic in temperate and tropical regions of the Americas, Africa and Europe, can lead not only to local dermonecrosis but also to serious systemic effects. At least 3 different *Loxosceles* species of medical importance are known in Brazil *(L. laeta, L. intermedia, L. gaucho)* and more than 3000 cases of envenomation by *L. intermedia* alone are reported each year. The therapy used for loxoscelism in Brazil is the administration of the anti-arachnidic serum, produced by the immunization of horses with the venoms from *Tityus serrulatus*, *Phoneutria nigriventer* and *Loxosceles gaucho,* by Butantan Institute. We have purified, characterized and cloned the toxins from *L. intermedia and L. laeta* venoms responsible for all the local and systemic effects induced by the whole venom. The main toxic component is endowed with sphingomyelinase D (SMase D) activity and various isoforms of this toxin are present in *Loxosceles* venoms. Recently, we have started to immunize horses with the recombinant form of SMases D*, i.e.,* two isoforms from *L. intermedia* (called SMases P1 and P2) and one from *L. laeta* (called SMase I), in order to produce a new anti-loxoscelic serum. **Objectives**: The aim of this study was to compare the neutralization potential of the new anti-loxoscelic and the anti-arachnidic sera, against the toxic effects of venoms from spiders of the *Loxosceles* genus of medical importance in Brazil**. Results**: The comparative analysis of the two anti-sera by Western blotting revealed that the anti-arachnidic serum is able to recognize the majority of the components present in the venoms of *L. intermedia*, *L. laeta* and *L. gaucho*. The new anti-loxoscelic serum, in contrast, recognized only components of 30-35 kDa, which correspond to the Mr of the native SMases D isoforms present in the *Loxosceles* venoms. By ELISA, it was determined that the anti-SMases D serum contains higher titers of IgGT and IgGa, b and c than the anti-arachnidic serum. Serum neutralization tests (*in vivo* and *in vitro*) showed that the anti-SMases D serum has better inhibitory activity against the toxic activities of the venoms from *L. intermedia* and *L. laeta*, such as sphingomyelinasic, dermonecrotic and hemolytic activities than the anti-arachnidic serum. For *L. gaucho* venom, the results were similar or, in some cases, better by using the anti-arachnidic serum. **Discussion** These data suggest that, although the new anti-loxoscelic serum shows a significant neutralization potential, it is still necessary to include in the immunization formulation a SMase D isoform from *L. gaucho* venom, in order to obtain a fully neutralizing horse antiserum against the three main important *Loxosceles* spiders that cause accidents in Brazil.

Supported by FAPESP, CNPq

1.45 Antimicrobial peptides in toxin of *Nephilengys cruentata* spider

Grignoux F[1], Pierangeli N[1], Portaro FCV[2], Camargo ACM[1], Silva Jr PI[1]
[1]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, [2]Laboratório de Imunoquímica, Instituto Butantan, SP, Brazil

Introduction: Antimicrobial peptides are ubiquitously produced throughout the phylogenetic tree. The widespread occurrence of these antimicrobial substances suggests that they play a key role in innate immunity against microorganisms and other pathogens. Despite the diversity in their structure, most of these antimicrobial substances are small (less than 10 kDa) cationic and amphipathic peptides. Over the past 20 years, several of these antimicrobial peptides were isolated from the hemolymph and toxins of invertebrates including chelicerates. Some antimicrobial peptides have been identified in arachnid toxins, mainly spider and scorpion. **Objectives:** Infectious diseases are one of the main causes of death in the human population. For the most part, it is due to the resistance of microorganisms to different antibiotics. Therefore, the search for antimicrobial compounds in the Brazilian fauna and flora could result in precious findings. The objective of this study was to identify antimicrobial factors in the toxin from the spider *Nephilengys cruentata*. **Methods:** *N. cruentata* is a nephilid spider found in tropical and subtropical Africa and several limited areas of South America (Brazil, Colombia and Paraguay), where it was probably introduced by humans. In São Paulo, these spiders are found commonly in gardens, along the edge of house roofs and the Atlantic Forest. The poison glands were removed and homogenized in water. Debris was removed with centrifugation at 1,600 x g for 10 min at 4 °C, and the supernatant used to isolate the toxins. We add acidified water (0.05% TFA) to the supernatant and submitted this material directly to pre purification by solid-phase extraction. The toxin in acidified water was loaded onto classic Sep-Pak C18 cartridges. Three stepwise elutions were successively performed with 5%, 40%, and 80% acetonitrile (ACN/0.05% TFA). The Sep-Pak fractions were concentrated in a vacuum centrifuge, reconstituted with 0.05% TFA, and submitted to reversed phase chromatography on a semi-preparative Jupiter C18 column. Elution was performed with a different linear gradient of ACN/0.05% TFA over 50 min at a flow rate of 1.5 mL/min. The active fractions were further purified to homogeneity by reversed-phase chromatography on an analytical Jupiter C18 column. Elution was performed using different linear biphasic gradients of acetonitrile in acidified water at a flow rate of 1.0 mL/min. The column effluent was monitored by absorbance at 214 nm. Fractions corresponding to absorbance peaks were hand collected, concentrated under vacuum, and reconstituted in Milli-Q water. The presence of antibacterial activity was determined by a liquid growth inhibition assay against Gram-negative bacterium *Escherichia coli SBS363,* Gram-positive bacterium *Micrococcus luteus A270* and yeast *Candida albicans.* **Results and Discussion:** We detected eleven fractions with activity against *Candida albicans* and ten fractions against *M. luteus* from Sep-Pak ACN 5%; seven fractions with activity against *C. albicans* and *E. coli* from Sep-Pak ACN 40%, and three fractions with activity against *C. albicans* from Sep-Pak ACN 80%. These fractions will be submitted to new activity assays and the molecular masses will be determined by MALDI-TOF spectrometry analysis.

Supported by FAPESP, CNPq

1.46. Antimicrobial peptides in the hemolymph of the mygalomorph spider *Acanthoscurria rondoniensis*

Chaparro-Aguirre E[2], Silva Jr PI[1]
[1]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, Instituto Butantan, SP, Brazil; [2]Universidade de Los Andes, Bogotá, Colômbia

Introduction: Arthropods defend themselves against invading microorganisms and parasites through cellular and humoral immune systems. The cellular reactions are carried out by the blood cells, which immobilize the invaders by phagocytosis and/or encapsulation. The humoral response is carried out by constitutive and inducible factors in the hemolymph. These molecules may be involved in recognition, facilitation of cellular immune response or direct antimicrobial action. Despite the diversity in their structure, most of these antimicrobial substances are small (less than 10 kDa) cationic and amphipathic peptides. Over the past 20 years several of these antimicrobial peptides were isolated and characterized from the hemolymph and toxins of invertebrates including chelicerates. **Objectives:** The objective of this study was to identify antimicrobial peptides in the hemolymph from the spider *Acanthoscurria rondoniensis*. **Methods:** The hemolymph from spiders was collected from prechilled animals by cardiac puncture. To avoid hemocyte degranulation and coagulation, the hemolymph was collected in the presence of sodium citrate buffer. The hemocytes were removed from plasma by centrifugation. Entire hemocytes were washed once with sodium citrate buffer and lysed by concentration in a vacuum centrifuge. After concentration, the hemocytes collected from the hemolymph were homogenized in a Dounce apparatus in 2M acetic acid. The supernatant obtained by centrifugation was directly subjected to pre-purification by solid-phase extraction. The acid extract was loaded onto serially linked classic Sep-Pak C_{18} cartridge. Three stepwise elutions were successively performed with 5%, 40%, and 80% acetonitrile in acidified water. The 40% Sep-Pak active fraction was concentrated in a vacuum centrifuge, reconstituted with MilliQ water, and submitted to reversed-phase chromatography on a semi preparative Jupiter C_{18} column. Elution was performed with a linear 2-60% gradient of acetonitrile in acidified water over 120 min at a flow rate of 1.5 mL/min. The column effluent was monitored by absorbance at 225 nm and the antimicrobial activity was determined by liquid growth inhibition assay against bacteria and yeast. **Results and Discussion:** In the process of isolating antimicrobial peptides from the tarantula spider *A. rondoniensis*, we found six fractions with antimicrobial activity after reversed-phase chromatography of the 40% ACN Sep-Pak fraction, arising from the pre-purification of an acidic extract of spider hemocytes. The peptides displayed activity against *Escherichia coli*, *Micrococcus luteus* and *Candida albicans*. Molecular mass determination of the two major activity peaks revealed that the active components present were very similar to mygalin (417 Da) and acanthoscurrin (two isoforms of 10,111Da and 10,225 Da) of the spider *A. gomesiana* hemocytes. The characterization of these peptides are still in progress. The investigation of these molecules and the understanding of their mechanisms of action may provide greater knowledge of the immune system of arachnids and arthropods. Moreover, the characterization of new molecules with antimicrobial activity may contribute to the development of new antibiotics.

Supported by Fapesp, CNPq

1.47 Antimicrobial peptides in the hemolymph of *Heterophrynus longicornis* (ARACNHIDA, AMBLYPYGI)

Ramos WR[1], Silva Jr PI[2], Camargo LMA[3]
[1]Universidade Federal de Rondônia, RO, Brazil; [2]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, Instituto Butantan, Brazil; [3]Instituto de Ciências Biomédicas V, Universidade de São Paulo, RO, Brazil

Introduction: Antimicrobial peptides are important components of the immune system of vertebrates and invertebrates and, in the last 20 years, many of these molecules were isolated and characterized in several organisms. The immune system of the invertebrates does not possess immunologic memory and no immunoglobulin; therefore, the defense against microorganisms is accomplished by hemocytes and other substances such as antimicrobial peptides. This study not only affords a better understanding of the immune system of arachnids and other arthropods, but can also contribute to the production and use of new drugs in medicine and agriculture, through the description and characterization of new molecules with antimicrobial activity. **Objectives:** The objective of this study was to identify the chemical structure and biological activity of antimicrobial peptides of the hemolymph of the Whip-spider *Heterophrynus longicornis*. **Methods:** The specimens were collected in the forest located on the Campus José Ribeiro Filho of the Federal University of Rondônia. The specimens were collected using active search. The hemolymph was collected with the animals previously cooled, by puncture of the dorsal vessel using an apyrogenic syringe with sodium citrate buffer. The hemocytes were separated from the plasma by centrifugation, and the plasma and the hemocytes which were washed separately in sodium citrate buffer, were submitted to acid extraction. The acid extract of the plasma and the hemocytes were submitted to a pre-purification in Sep-Pak C18 and then, the column eluted with different concentrations of acetonitrile (5%, 40% and 80% ACN). The fraction containing 40% ACN was subjected to reversed-phase chromatography (RP-HPLC), using a semi-preparative Jupiter C18 column, where the absorbance of the effluent was monitored at 225 nm and the antimicrobial activity was determined by inhibition assay in liquid culture against *Escherichia coli*. **Results and Discussion:** The fractions obtained from plasma and hemocytes, showed antimicrobial activity against *E. coli*. Elution of 40% ACN yielded four plasma peptides, and three peptides from the hemocytes inhibited the growth of the Gram-negative bacterium *Escherichia coli* SBS363. New assays will be developed using other microorganisms, using the fractions that showed activity against *E. coli* and the fractions also obtained starting with elution using 5% and 80% ACN. All molecules found with antimicrobial activity will be sequenced and the activity spectrum of each one will be determined.

Supported by FAPESP, CNPq

1.48 Antimicrobial peptides in Myriapods

Chaparro-Aguirre E[2], Silva Jr PI[1]
[1]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, Instituto Butantan, SP, Brazil; [2]Universidade de Los Andes, Bogotá, Colômbia

Introduction: Arthropods constitute one of the oldest groups of organisms, and also show a wide distribution in different ecosystems and habitats. This fact leads to the question about which factors allowed such an ecological success in these organisms. Knowing that most of the environments where they are found show a high presence of microorganisms and pathogens, it can be affirmed that part of the success of arthropods in colonizing these environments are their defense mechanisms and immune systems. One of the main components of the defense mechanism in vertebrates and invertebrates are the peptides with antimicrobial functions, which control the growth and invasion of the different pathogens. For this reason, it is important to study this, not only to understand the success of these invertebrates and their defense mechanisms, but also to find alternatives to fight infectious diseases that affect humans. This is why the purification and characterization of these antimicrobial peptides and the knowledge of the function of their immune systems becomes more interesting. **Objectives:** In this study, the objective was identify antimicrobial factors in the hemolymph from three different species of Myriapods that are widely distributed in Brazil and Colombia. **Methods:** The hemolymph was extracted from three different species: *Scolopendra viridicornis***,** *Scolopendra gigantea* and *Otostigmus* sp**.** Plasma and hemocytes were submitted to acid extraction and fractionated in two steps. First, using C18 Sep Pak column cartridge in three stepwise elution with 5, 40 and 80% acetonitrile (ACN) in trifluoroacetic acid (TFA), 0.05%. All the fractions were concentrated in a vacuum centrifuge, reconstituted in 0.05% TFA and loaded onto a semi-preparative C18 Jupiter column using a linear gradient of ACN in 0.05% TFA for the second purification step. The column effluent was monitored by absorbance at 225 nm and the antimicrobial activity was determined by liquid growth inhibition assay. **Results and Discussion:** After RP-HPLC, activity was detected against the microorganisms used in the inhibition assay, in the fractions from hemocyte and plasma acid extract eluted with 5, 40 and 80% ACN. Also, after comparing the different profiles obtained with HPLC, it was observed that the immune system of myriapods is not established in early instars, but prior stimuli are required for the production of the antimicrobial peptides. In spite of this, the fact that in some cases, the animal was not stimulated before the extraction was performed did not impede the finding of some antimicrobial activity. In the hemolymph of *S. viridicornis,* a peak with a molecular mass of 4,307 Da was found in the hemocyte fraction eluted with 40% ACN, which showed activity against the bacteria *M. luteus* and *E. coli*. The purification and characterization of these peptides are still in progress.

Supported by FAPESP, CNPq

1.49 Antimicrobial peptides in *Bothrops jararaca* venom

Ayroza G, Camargo ACM, Silva Jr PI
Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, Instituto Butantan, SP, Brazil

Introduction: Due to the development of antibiotic resistance in microorganisms, antimicrobial peptides from natural sources have attracted attention in recent times, in order to find new therapeutic agents. Several antimicrobial peptides have been isolated from a wide range of animal sources. Antimicrobial peptides (AMPs) are an extremely diverse group of small proteins that are considered together, because of their native antimicrobial activity and because their function is essential to the animal immune response. Snake venoms are a complex mixture of components, which have a diverse array of actions both on prey and human victims. Many of these components are biologically active proteins that function to kill or immobilize the prey. Investigators in the fields of toxinology and medicine have long appreciated the complexity of *Viperidae* venoms. *Bothrops* venoms in general contain proteolytic enzymes (metalloproteinases and serine proteinases), desintegrins, phospholipase A2, type C lectins, CRISPs and L-amino acid oxidase. **Objective:** The objective of this study was to identify new antimicrobial peptides from *Bothrops jararaca* venom. **Methods:** The lyophilized venom was reconstituted with 1mL of acidified water (TFA - trifluoroacetic acid, 0.05%), centrifuged and the soluble part was subjected to HPLC reversed-phase chromatography on a semi-preparative Jupiter C18 column. Elution was performed with different linear gradients of ACN/0.05% TFA over 60 min at a flow rate of 1.5mL. The presence of antibacterial activity was determined by a liquid growth inhibition assay against the Gram-negative bacteria *Escherichia coli SBS363*, Gram-positive bacteria *Micrococcus luteus A270* and yeast *Candida albicans*. **Results:** According to our results, three fractions inhibited the growth of *Micrococcus luteus A270*, two fractions showed activity against *Escherichia coli SBS363* and six fractions inhibited the growth of *Candida albicans.* These fractions will be re-purified on an analytical column and submitted to new activity assays. The molecular masses of the fractions obtained from reversed-phase chromatography will be determined by MALDI-TOF spectrometry analysis.

Support by FAPESP, CNPq

1.50 Self-proteolysis regulation in the *Bothrops jararaca* venom: The metallopeptidases and their intrinsic peptide inhibitor*

Marques-Porto R[1], Pimenta DC[2]
[1]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, [2]Laboratório de Bioquímica e Biofísica, Instituto Butantan, SP, Brazil

Introduction: Snake venom proteome variation is a well-documented phenomenon, whereas peptidome variation is still relatively unknown. Although snake venom metallopeptidases have long been known from the biochemical up to the clinical point of view, the mechanisms by which these enzymes are regulated in the reptile's venom gland remain only speculative. **Objectives:** We used a biological approach to explore the inhibitory activities present in the whole venom of *Bothrops jararaca* that prevents venom self-proteolysis and/or digestion of the glandular tissue. **Methods:** Reversed-phase HPLC, mass spectrometry (including de novo sequencing) and enzymatic activity profiling were employed accordingly. **Results:** We demonstrated successfully that there are three synergistic weak inhibitory mechanisms that are present in the crude venom that are able to abolish the metallopeptidase activity in situ, namely: (i) calcium citrate chelation; (ii) acidic pH and; (iii) enzymatic competitive inhibition by the tripeptide Pyroglutamyl-lysyl-tryptophan (<EKW). **Discussion:** Taken together, these three factors establish an environment that strongly inhibits the crude venom metallopeptidase activity as well as a purified metallopeptidase from this same venom. However, this inhibition can be totally reversed by dilution into an optimal pH solution, such as the blood.

Supported by FAPESP
***This work was developed by the authors when working at the CAT/CEPID. Instituto Butantan**

1.51 Serine-protease inhibitors from *Nephilengys cruentata* spider venom gland

Pierangeli N[1], Silva Jr PI[1], Camargo ACM[1], Chaparro E[1], Portaro FCV[2]
[1]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, [2]Laboratório de Imunoquímica, Instituto Butantan, SP, Brazil

Introduction: Biomolecular inhibitors that blocked specifically proteolytic enzymes are of interest in basic and applied research. Peptides can modulate enzymatic activity by acting as purely competitive inhibitors or as activators of proteolytic enzymes. In the present work we searched for new, specific and potent biological compounds able to inhibit or enhance the proteolytic activity of the serine proteases trypsin and thrombin. **Objectives:** The aim of the study was to identify and characterize natural serine-protease inhibitors present in the *Nephilengys cruentata (NC)* spider venom gland, a common little spider that lives near lights of houses and gardens. **Methods:** The venom glands from ten (10) female *NC* spiders were obtained and submitted to toxin extraction through 2 steps. First, using C18 Sep Pak column cartridge in three stepwise elution with 5, 40 (V40) and 80% acetonitrile (ACN) in 0.05% trifluoroacetic acid (TFA). Afterward, the fractions were separated in a semi-preparative reversed-phase column using a linear gradient of ACN. The peaks were collected manually for the trypsin and thrombin inhibition studies. The substrate Abz-FRSSRQ-EDDnp was used for both enzymes. **Results:** The inhibition of trypsin and thrombin by the V40 fraction was initially observed, demonstrating strong activity. The peaks obtained from V40 were assayed for activity. The new HPLC step revealed two inhibitory fractions, with apparent homogeneity (single peaks). **Discussion:** The results suggest the existence of compounds in the *NC* spider venom gland capable of blocking serine proteases. The peaks showing inhibitory activity will be submitted to mass spectrometry analysis to determine their primary sequence. Thus, we believe that some selective inhibitors for these enzymes could be found using the present methods.

Supported by CAT-CEPID/FAPESP, UNIEMP, CNPq

1.52 New trypsin inhibitor isolated from a Microcystis bloom in the Violão Lagoon, Southern Brazil

Pierangeli N[1], Sant'Anna CL[2], Carvalho LR[2], Rioli V[1], Konno K[1], Portaro FCV[3]
[1]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, [3]Laboratório de Imunoquímica, Instituto Butantan, SP, Brazil; [2]Seção de Ficologia, Instituto de Botânica, SP, Brazil

Introduction: Cyclic heptapeptides known as microcystins, which have the general structure cyclo - (D - Ala -L-X-D-eryythro-methyl-iso-Asp-L-Y-Adda-D-isoGlu-N-methyl-dehydro-Ala) where X and Y are variable L-amino acids, are responsible for the potent hepatotoxicity of certain species of cyanobacteria (blue-green algae). Microcystins, of which over 65 structural variants are known, are cyclic heptapeptides, which include potent hepatotoxins and tumor promoters. They are produced by cyanobacteria in aquatic and terrestrial environments and have been involved, or implicated, in acute and chronic health problems in animals and humans. Over fifty patients at a dialysis center in Caruaru, Brazil, died in 1996 from intravenous exposure to cyanotoxins, primarily microcystins. **Objectives**: In the present study, we screened extracts of a natural bloom that occurred in Violão Lagoon, for their potential to inhibit trypsin-like enzymes. **Materials and Methods**: Microcystis sp. bloom samples were collected from Violão Lagoon and the extraction of microcystin for purification was performed with 1 g of lyophilized material using 5% (v/v) aq. HOAc. The purification of the inhibitor was subsequently by reversed-phase HPLC (Shimadzu, class VP) with a linear gradient of 5% to 95% acetonitrile/H_2O/0.1% TFA at a flow rate of 2.5 ml/min over 30 min. The peaks were manually collected and screened through the use of FRET substrate for serine peptidases (ABZ-FRSSRQ-EDDnp). All mass spectra were acquired on a Voyager Elite MALDI/TOF/MS (Applied Biosystems, Foster City, CA, USA) equipped with a delayed extraction source and 337 nm pulsed nitrogen laser. The accelerating voltage was 20 kV. Argon gas was used for the CID/PSD experiment. A Matrix, a-cyano-4-hydroxycinnamic acid (Aldrich) was prepared at a concentration of 10 mg/ml in 1:1 CH_3CN/0.1%TFA. The sample (0.5 ml) dropped onto the MALDI sample plate was added to the matrix (0.5 ml) and allowed to dry at room temperature. **Results and Discussion**: Although cyanobacteria produce many kinds of non-toxic peptides, the trypsin inhibitor screened here, to our surprise, was found to be a new microcystin. Thus, we report here, for the first time, that a new microcystin-LR was discovered and that this peptide is also a potent inhibitor of trypsin (Ki = 2.1 μM) and thrombin (Ki = 1.2 μM). Altogether, the hepatotoxicity and inhibitory effects on serine peptidases of the novel microcystin observed in the present study, can explain more clearly the high toxicity of the molecule.

Supported by CAT/CEPID- FAPESP, UNIEMP, CNPq

1.53 Screening of the Botanic Institute Cyanobacteria Bank: mouse acute toxicity and *in vitro* cytotoxicity

Augusto NF[1], Carvalho LR [2], Sant'Anna CL[2], Machado-Santelli GM[1], Rangel M[3]
[1]Departamento de Biologia Celular e Desenvolvimento, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil; [2]Departamento of Ficologia, Instituto de Botânica, SP, Brazil; [3]Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil

Introduction: Cyanobacteria are known as excellent sources of vitamins and proteins, which are commercialized all around the world. They are an important source of fine chemistry products, renewable fuel and bioactive compounds. It is towards this last concern that research has been directed recently, since some isolated compounds show antiviral, anticancer and antibacterial properties as well as other activities. Among the cyanobacteria secondary metabolites are the endotoxins, produced by members of the orders Nostocales and Chroococcales, including species from the genus *Anabaena, Aphanizomenon, Microcystis* and Nodularia. For humans, ingestion of these endotoxins causes symptoms such as gastroenteritis, nausea, vomiting, diarrhea, abdominal pain, fever, eye and skin irritation, damage to liver and kidneys, etc. Deaths have already occurred in Brazil due to ingestion of freshwater contaminated with hepatotoxic cyanobacteria (*Microcystis*), and dermatitis is caused by *Lyngbya majuscula.* **Objectives**: In the present work, we studied extracts of cyanobacteria from the Botanical Institute Algae Bank, whose lab growth has been established, and also algae blooms. Our aims were to identify, through toxicological assays, toxin-producing cyanobacteria and to determine the cyanobacteria extracts that have antiproliferative activity against human breast cancer cells (MCF-7). **Materials and Methods**: Extract preparation: cells were filtered (filter AP-20), lyophilized and extracted (4x) with 0.1M acetic acid and sonication (4 x 10 sec, 50 W). The extracts were freeze-dried and kept frozen (-20°C) until use. For the toxicity assays, male Swiss mice (20-25g) were used. The extracts were injected (i.p.) and the symptoms exhibited by the animals were recorded for up to 36 h. In the antiproliferative assays, the MCF-7 human breast cancer cell line was used. The cells were grown in Sigma culture medium (DMEM) with 10% fetal bovine serum (Cultilab) in cell culture microplates (96-well). Cell viability was accessed using the Cell Titer 96^{TM} kit (Promega®) and the resulting absorbances were measured in an ELISA plate reader at 570nm. **Results and Discussion**: In the toxicity experiments, 24 strains and 2 algae blooms were tested. Microcystins (hepatotoxins) were detected in seven strains (all *Microcystis* spp.) and in "Lagoa do Violão" bloom. One strain contained saxitoxin (*Raphidiopsis brookii*), and another strain had anti-cholinesterase activity (*Aphanothece bachimanii*). In the cytotoxicity assays, 13 strains and one algae bloom (from "Lago Guaíba") were tested. Four strains displayed significant (over 25%) cytotoxicity against breast cancer cells at 30 µg/ml concentration: *Raphidiopsis brookii* (338), *Planktothrix* sp. (370), *Cylindrospermopsis* sp. (811), and *Microcystis aeruginosa* (856). Dose-response curves of strains 370 and 811 are currently being determined. This work represents an effort from three different institutions: Botanical Institute, Butantan Institute and University of Sao Paulo to study the pharmacological properties of almost 250 strains from the Botanical Institute Cyanobacteria Bank, and the toxicity of the cyanobacteria blooms that are considered a public health problem in Brazil.

Supported by Fapesp, CNPq

1.54 Ionotrophic and metabotropic glutamate antagonists on the behavioral, eletroencephalographic, and neuropathological effects induced by TsII isolated from *Tityus serrulatus* scorpion venom

Oliveira DA[1], Lebrun I[2], Sandoval MRL[1]
[1]Laboratório de Farmacologia, [2]Laboratório of Bioquímica e Biofísica, Instituto Butantan, SP, Brazil

Introduction: TsII, a β-toxin, binds to site 4 of Na+ channels and induces an increase in glutamate release in the central nervous system. Intrahippocampal injection of this toxin induces behavioral and electroencephalographic seizures, and hippocampal lesions. We postulated that a glutamate increase in the hippocampus could be responsible for the neurotoxicity induced by TsII. **Objectives:** In the current work, we studied the ionotrophic and metabotropic glutamate receptor antagonists with the aim of blocking the mainly neurotoxic effects induced by TsII. **Methods:** Four days after a stereotaxic implantation of a hippocampal guide cannula and depth electrodes the rats were injected i.p. with MK-801 (0.5 or 1 mg/kg) or intrahippocampal with CNQX (2.0 μg/μl), AP-3 (0.08 or 0.04 mg/μl) or MCPG (0.09 or 0.18 mg/μl) 30 min before TsII, 2μg/ul, injection into the hippocampus. A group of rats was injected only with one of the glutamate antagonists. EEG and animal behavior were recorded for 8 h. After 7 days, the brains were processed for morphological analyses. **Results:** The results obtained with the ionotropic glutamate antagonist MK-801 (0.5 mg/kg), a noncompetitive antagonist of NMDA receptor and CNQX (2.0 μg/μl), a competitive antagonist of AMPA receptors, showed that these drugs administered before TsII were able to inhibit behavioral seizures and reduced the epileptic discharges and cluster spikes. These antagonists prevented the development of the neuronal loss induced by TsII ($p<0.05$, ANOVA followed by Dunnett's test). MCPG (0.09 mg/ul) injected before the toxin blocked the behavioral seizures, but staring and agitation were still observed. The long lasting epileptic discharges were prevented, but the presence of spikes and short epileptic discharges were recorded. Concerning the neurodegeneration induced by TsII, this antagonist was not able to prevent neuronal loss. AP-3, an antagonist of group-I of metabotropic glutamate receptor, was not able to protect the animals from the mainly neurotoxic effects. **Discussion:** The pre-treatment of the rats with ionotropic glutamate antagonists, MK-801 or CNQX, was able to block the behavioral and electrographic seizures and the neuronal loss induced by TsII. However, the pre-treatment of the rats with the metabotropic glutamate antagonists MCPG partially blocked epileptic discharges but it was not able to prevent the neurodegeneration induced by TsII. Previously, we demonstrated that TsII increases glutamate release into the hippocampus. Now, we showed that glutamate antagonists can reduce or abolish the neurotoxicity induced by TsII. These data confirm the participation of the glutamate in the neurotoxicity induced by TsII. The persistence of neuronal damage mainly in those animals treated with the metabotropic antagonists could be due the involvement of other neurotransmitters released in the hippocampus by the toxin, such as acetylcholine.

Supported by FAPESP, Fundação Butantan

1.55 Hippocampal effects of some doses of toxin IV-IV isolated from *Tityus serrulatus* scorpion venom: a behavioural, eletroencephalografic and histophatologic study

Freitas MM[1,3], Dorce VAC[1], Lebrun I[2], Nencioni ALA[1]
[1]Laboratório de Farmacologia, [2]Laboratório de Bioquímica e Biofísica, Instituto Butantan, SP, Brazil; [3]Coordenadoria de Controle de Doenças – SES, SP, Brazil

Introduction: Scorpion venoms contain, among other substances, neurotoxins that act on ion channels, mainly sodium and potassium. Previous studies showed that some toxins of *Tityus serrulatus* venom (vTs), such as TsTX, have epileptic and neurotoxic effects when injected into the hippocampus of the rats. **Objective:** The aim of this study was to investigate the effects of IV–IV toxin isolated from this venom in the hippocampus of rats. **Methods:** Male Wistar rats (220 – 250 g) were anesthetized and positioned in a stereotaxic frame. Stainless steel guide cannulas and bipolar twisted electrodes were chronically implanted in the hippocampus. One day after surgery the animals were injected with 0.5, 0.75 or 1 µg/µl of toxin (n=6) or Ringer solution (control group, n=6). After the injections, continuous electroencephalographic recording (EEG) and observations of animals' behavior were performed for periods of 4h. Seven days after the injections, the animals were sacrificed and perfused. The brains were removed and prepared for histological analysis. **Results:** Animals injected with 0.5 µg/µl showed a little discomfort followed by quick "wet dog shakes." EEG displayed a moderate discharge. At the end of the EEG, all animals remained sleeping. Animals injected with the dose of 0.75 µg/µl showed discomfort, wet dog shakes and yawning. EEG displayed intense and moderate discharges. Animals injected with 1 µg/µl the EEG showed intense epileptic-like discharge, often accompanied by behavioral alterations, such as wet dog shakes and myoclonus. The histopathological analysis showed neuronal death in CA1 ipsilateral and contralateral areas with all doses of the toxin, and in CA3 ipsilateral area in animals injected with 1 µg/µl. **Discussion:** Due to the likeness of effects between the toxin IV–IV and the toxin TsTX, we conclude that toxin IV-IV causes an increased release of neurotransmitters, mainly glutamate, in the central nervous system causing a convulsive effect and neuronal death.

Supported by Fundação Butantan, CCD/SES - SP

2. Microorganisms and vaccines

2.01 Analysis of the immune response induced by mucosal immunization of mice with *Lactobacillus casei* expressing enteropathogenic *Escherichia coli* ß-intimin fragments

Ferreira PCD, Campos IB, Ho PL, Oliveira MLS
Centro de Biotecnologia, Instituto Butantan, SP, Brazil

Introduction: Enteropathogenic *Escherichia coli* (EPEC) is a cause of significant morbidity and mortality among infants in developing countries. One of the virulence factors that promote the intimate adhesion of EPEC with enterocytes in the small intestine is intimin, a 94kDA outer membrane protein, essential for full virulence. The adhesion results in activation of several signaling proteins in the host cells and rearrangement of the cytoskeleton, leading to the formation of attaching and effacing lesions (A/E lesions) and disruption of the cell membrane. Based on antigenic variations, intimins are classified in several subtypes that can be grouped into nine families; among them, ß-intimin is one of the most frequently found subtype in clinical isolates. Lactic acid bacteria are commensal microorganisms present in the gastrointestinal mucosa of healthy individuals that have been used as carriers for antigen presentation in different models. **Objectives:** The aim of this work was the comparative analysis of the immune responses induced by lactic acid bacteria expressing fragments of ß-intimin, through oral and nasal immunization of mice, towards the development of a mucosal vaccine against EPEC infections. **Methods:** The ß-intimin fragments were cloned into a vector based on the *Lactococcus lactis* P1 promoter. The recombinant vector was used for transformation of *L. lactis* and *Lactobacillus casei*. Intracellular expression of the fragments in *L. lactis* and *L. casei* extracts was analyzed by Western blotting. The lactobacilli were tested for the induction of the immune system upon oral and nasal immunization of C57B/6 mice. The anti-intimin antibody titers were determined by ELISA. **Results and Discussion:** The results indicate that recombinant lactobacilli were able to induce a systemic and mucosal specific immune response against the antigen, suggesting this to be a promising strategy for the development of a mucosal vaccine against EPEC.

Supported by FAPESP, CNPq, Fundação Butantan

2.02 Construction of pseudoparticles containing E1 and E2 hepatitis C virus proteins

Urbano PRP, Lemos MAN, Jorge SAC, Pereira CA
Laboratório de Imunologia Viral, Instituto Butantan, SP, Brazil

Introduction: Hepatitis C is a serious problem in public health. The hepatitis C virus does not replicate in cell culture, which makes virological studies for vaccine production difficult. Thus, new studies in the attempt to understand the formation of the viral particle of HCV and its infection are necessary, aiming to study mechanisms of HCV infection and to create strategies of inhibition of the entrance of HCV into the cells. Along this line, some viral particles (VLPs "virus-like particles") have been produced in cell cultures containing different viral proteins of the different viruses. **Objectives**: In this work, we intend to demonstrate the penetration of pseudoparticles containing E1 and E2 envelope glycoproteins of HCV and the RNA of GFP fluorescent protein in monkey cells (VERO) and in macrophages. **Methods:** The pseudoparticles were obtained through co-transfection of HEK-293 cells with 3 genetic vectors: a) containing the MLV gag and pol genes, b) the GFP gene (fluorescent protein) and c) HCV E1 and E2 genes. The relation of the proteins E1 and E2 in the penetration of the pseudovirus was examined by the fluorescence emitted in VERO cells infected with pseudoparticles due the GFP expression. **Results:** The 3 genetic vectors were constructed and inserted into the HEK293 cells. The pseudoparticles produced in HEK 293 cells were used for infection of VERO cells, but in preliminary experiments infected VERO cells failed to show fluorescence in flow cytometry. **Discussion:** The lack of fluorescence in infected VERO cells may be due to the low quantities of pseudoparticles produced in HEK293 cells. New experiments are planned for quantification of pseudoparticles.

Supported by CAPES (COFECUB Program)

2.03 Purification of capsular polysaccharide of *Haemophilus influenzae* type b by enzymatic hydrolysis and tangential ultrafiltration membrane in the presence of detergents deoxycholate and betaine

Albani SMF[1], da Silva MR[1], Pradella JGC[1], Tanizaki M[1], Zangirolami TC[2], Takagi M[1], Cabrera-Crespo J[1]

[1]Centro de Biotecnologia, Instituto Butantan, SP, Brazil; [2]Departamento de Engenharia Química, Universidade Federal de São Carlos, SP, Brazil

Introduction: The bacterium *Haemophilus influenzae* type b is a pathogenic Gram-negative responsible for respiratory tract infections, meningitis and pneumonia, especially in children younger than 5 years old. The capsular polysaccharide (PS) from this microorganism, which is considered the most important virulence factor, is currently purified, conjugated to a protein and used as a vaccine against *Haemophilus*. The classical purification processes of these polysaccharides are very complex, including many precipitations with organic solvents, phenol extraction and centrifugation/ultracentrifugation steps in toxin- and explosion-proof installations. **Objective**: This work focuses on reducing or replacing the organic solvent precipitations and the elimination of phenol by tangential ultrafiltration with enzymatic hydrolysis. The elimination of potentially toxic and explosive purification steps will reduce the fixed capital investments in appropriated building and reduce the work in an unhealthy environment. **Methods:** The enzymes used in this work were: nuclease (benzonase®) and proteases (nagarse and trypsin), respectively. The enzymatic hydrolysis (EH) was performed on a bioreactor cellulose or polyether sulfone membrane of 100 kDa, with diafiltration after the use of each enzyme. Final diafiltration was carried out in the presence of deoxycholate and betaine. **Results:** With a membrane of regenerated cellulose we obtained a RP_{PRT} =31 and RP_{NA} =18, but with recovery of 13% of PS (RP_{PRT} - relative purity of PS with respect to protein; RP_{NA} - relative purity of PS with respect to nucleic acid; RP_{PRT} = mg PS/mg PRT; RP_{NA} = mg PS/mg NA). With a membrane of polyether sulfone, the relative purity was RP_{PRT}= 25 and RP_{NA} =31, and 60% of PS recovery. One more purification step was added with diafiltration in 100 kDa with the zwitterionic detergent betaine. After this step, we reached a RP_{PRT}= 55 and RP_{NA} =136, and 41% of PS. **Discussion:** The purification using a membrane of regenerated cellulose had a low recovery of PS. The addition of betaine improves the purification process with a fair recuperation when in conjunction with the polyether sulfone membrane. The use of a surfactant such as deoxycholate (positively charged) and betaine (zwitterionic) is necessary, as both detergents work in different ways resulting in a synergistic effect in this process.

Supported by FAPESP
Acknowledgment: Albani SMF is on partial leave from São Paulo State Secretary of Education

2.04 New whole cell pneumococcal vaccine protects mice against pneumococcal nasopharyngeal colonization

Silva APGB[1], Sbrogio-Almeida ME[1], Liberman C[1], Takagi M[1], Gonçalves MV[1], Malley R[2], Anderson P[3], Leite LCC[1], Dias WO[1]

[1]Centro de Biotecnologia, Instituto Butantan, SP, Brazil; [2]Childrens Hospital and Harvard Medical School, Boston, USA; [3]University of Rochester, New York, USA

Introduction: The infections caused by *Streptococcus pneumoniae* are responsible for ten million deaths every year around the world. The currently available pneumococcal vaccines either offer low protection for the high-risk groups or are too expensive for general vaccination in developing countries. **Objectives:** We studied the humoral immune response and protection of Balb/c and C57 Black mice, elicited by a whole cell pneumococcal vaccine, using cholera toxin (CT) or cholera toxin subunit B (CTB) as adjuvants. **Methods and Results:** The vaccine (WCPV) is an ethanol-killed, non-encapsulated mutant from *Streptococcus pneumoniae*, strain RX1AL- Kan^R Ply, derived from a serotype 2 strain, autolysin negative and carrying a kanamycin resistance gene. The WCPV administered intranasally in Balb/c mice, mixed with CT (1 μg/dose) or CTB (10 μg/dose) elicited higher serum IgG antibody titers, determined by ELISA, against vaccine antigens, when compared with the control group (IgG antibody titers of 288, 272 and 112, respectively). When C57 Black mice were employed, the vaccine with or without CT induced significantly higher serum IgG antibodies, when compared to the adjuvant alone (IgG antibody titers of 372, 192 and 0, respectively; Mann Whitney = $p < 0.05$). This preparation administered intranasally with CT or CTB showed a protective effect in Balb/c mice, against an intranasal challenge with capsulated pneumococci (strain 603/serotype 6B), significantly reducing nasopharyngeal colonization, in relation to the saline group (vaccine + CT = 7, vaccine + CTB = 196 and saline = 1475 CFU/animal, respectively; Mann Whitney = $p < 0.05$). When C57 Black mice were employed, the vaccine with CT significantly reduced nasopharyngeal colonization, in relation to the group injected with saline (61 and 1006 CFU/animal, respectively; Mann Whitney = $p < 0.05$). **Discussion:** Since it has been shown that WCV elicits protection against pneumococcal disease, cell-mediated immunity must be further investigated. Taken together, our results are promising, showing new perspectives for pneumococcal vaccines.

Supported by FAPESP, Fundação Butantan

2.05 Production of recombinant fPspA1 from *Streptococcus pneumoniae* in *Escherichia coli* high cell density cultivation

Csordas FCL, Sari RS, Ferreira DS, Giordano RC, Silva M, Lopes APY, Tanizaki MM, Cabrera-Crespo J, Pradella JGC, Gonçalves VM
Centro de Biotecnologia, Instituto Butantan, SP, Brazil

Introduction: *S. pneumoniae* is an important pathogenic bacterium that causes pneumonia, meningitis, otitis media and bacteremia. Conjugate vaccines are most effective, but their development involves high costs. The use of recombinant pneumococal surface protein A fragment (rfPspA1) as carrier may induce higher protection and cross reaction between serotypes. Cultivation to produce recombinant protein at high cell density (HCD) is essential for vaccine development, because the higher the amount of protein produced, the lower the cost of vaccine. **Objectives:** The aim of the study was: 1) to evaluate rfPspA1 production in minimum medium to reduce culture proteolytic activity; 2) to reach HCD using exponential feeding of substrate limiting growth; and 3) to investigate the viability to decrease the concentration of IPTG, an expensive and toxic reagent, by using lactose. **Methods:** The f*pspA*1 gene was cloned into pET37b^{+} and expressed in *E. coli* BL21(DE3). HCD medium with 25g/L glucose was used for cultivation (BioFlo 2000, 5L-reactor). The glucose and acid concentrations were analyzed by HPLC and the protein by 12% SDS-PAGE. Four schedules were tested: 1) batch cultivation and induction with 20g/L lactose and 0.1mM IPTG for 4 h; 2) fed-batch cultivation, induction with 0.1mM IPTG and feeding 20g/L lactose at 1ml/min for 12 h; 3) fed-batch cultivation and induction with 0.4mM IPTG and 20g/L lactose for 4 h; 4) the same as (3) but induction with 0.5mM IPTG and 20g/L lactose while feeding glucose at very low flow rate (0.05 h^{-1}). After cell disruption, the clarified supernatant was applied to Ni^{2+}- and Q-Sepharose columns for rfPspA1 purification. The proteolytic activity was determined using 1%(w/v) casein as substrate, which was incubated with samples at 37°C for 24 h, the reaction was interrupted with 50%(w/v) trichloroacetic acid, and the products were quantified in the supernatant by A_{280nm}. **Results:** The cell growth in batch phase reached A_{600nm}=32±2 (~9.6g/L) and at the end of fed-batch cultivations A_{600nm}=148-213 (~45g/L-64g/L). In the batch phase, the maximum specific growth rate (μ_{max}) was 0.51 ± 0.048 h^{-1} and the biomass yield factor was 0.33±0.013 g cell/g glucose. Although not seen by SDS-PAGE, the rfPspA1 was produced even when 0.1mM IPTG was used for induction of HCD culture (~300mg protein/L broth), as it was recovered in the purification process. The rfPspA1 production was favored when the IPTG concentration was increased to 0.5mM and the glucose was fed during the induction phase, reaching ~2g protein/L broth. The proteolytic activity was reduced when the minimum medium was employed and protease inhibitor was added to the lysis buffer. The residual proteolytic activity was eliminated in the non-absorbed fraction of Ni^{2+} chromatography. **Discussion:** The production of rfPspA1 was obtained in both batch and fed-batch cultivation. While full induction in batch cultivation was easily reached with only 0.1mM IPTG, for the induction at HCD it was necessary to use 0.5mM IPTG, 20g/L lactose and glucose feeding at low flow rate. This culture condition yielded 64g/L of cell and 2 g/L of rfPspA1. Using an appropriate downstream process may allow purifying the protein without the use of protease inhibitor and achieving high purity requirements at minimal cost.

Supported by FAPESP, CNPq

2.06 Optimized immune response elicited by pneumococcal surface protein A DNA vaccine is characterized by balanced IgG1:IgG2a ratio, IFN-γ and TNF-α production

Ferreira DM, Darrieux M, Oliveira MLS, Leite LCC, Miyaji EN
Centro de Biotecnologia, Instituto Butantan, SP, Brazil

Introduction: Diseases caused by *Streptococcus pneumoniae*, such as pneumonia, meningitis and bacteremia, are a major public health problem. The need to achieve broader serotype coverage at a lower cost has stimulated attempts to develop a vaccine based on conserved protein antigens of pneumococci such as PspA (pneumococcal surface protein A). We have previously shown that DNA immunization based on PspA was able to protect mice at levels similar to recombinant protein (with alum as adjuvant) despite lower antibody response. **Objective:** The present study aims to characterize the induction and functionality of the antibodies and cellular immune response induced by immunization against *S. pneumoniae* using PspA as DNA vaccine and recombinant protein. **Methods:** BALB/c and C57BL/6 WT mice, as well as IL-4 KOs, were immunized with DNA vaccine or recombinant protein (rPspA), and the anti-PspA antibodies induced were analyzed for their isotype profile, capacity to bind to the surface of intact pneumococci, as well as for the ability to mediate complement deposition. The profile of cytokines secreted by splenocytes of the immunized mice after challenge with virulent pneumococci was also analyzed. **Results:** Evaluation of anti-PspA antibodies showed that DNA vaccination elicited a balanced humoral response with the induction of similar levels of IgG1 and IgG2a, while recombinant protein showed preferential induction of IgG1. Anti-PspA antibodies obtained after rPspA immunization showed a more prominent binding than pSec-*pspA*3NS immunization in BALB/c and C57BL/6 in both WT and IL-4 KO mice. On the other hand, complement deposition assays showed that sera obtained from C57BL/6 WT and IL-4 KO mice immunized with pSec-*pspA*3NS mediated similar levels of complement deposition compared with rPspA3NS sera, while sera from BALB/c WT mice immunized with DNA vaccine were able to mediate complement deposition at higher levels when compared with rPspA, despite similar levels of IgG2a. We have also analyzed the induction of IFN-γ after stimulation of splenocytes with PspA *in vitro* and were able to detect a marked increase in number of Spot forming cells secreting IFN-γ by ELISPOT and in culture supernatant by ELISA after intraperitoneal challenge with virulent pneumococci only in mice immunized with the DNA vaccine. We have also looked for TNF-α and, interestingly, observed that only the pSec-*pspA*3NS immunized group showed increased amounts of this cytokine after challenge. **Discussion:** Our results show that immunization with both recombinant protein and DNA vaccine elicit immune responses, which are sufficient for protection against lethal challenge with *S. pneumoniae*, but that are qualitatively different. While higher amounts of total anti-PspA IgG elicited by recombinant protein led to increased antibody binding to the pneumococcal surface, the balanced IgG1/IgG2a ratio elicited by DNA vaccination showed a correlation with efficient complement deposition. Furthermore, the cellular immune response elicited by DNA vaccination was characterized by secretion of Th1 cytokines. The response elicited by the DNA vaccine seems to be qualitatively more adequate than recombinant protein.

Supported by FAPESP, Fundação Butantan, Millenium Institute-Gene Therapy Network (MCT-CNPq)

2.07 New pertussis vaccines for developing countries

Prestes AFRO[1], Horton DSPQ[2], Sakauchi MA[3], Kubrusly FS[1], Iourtov D[1], Furuyama N[3], Dias SC [1], Quintilio W[2], Santos FL[3], Darini E[3], Prado SMA[3], Higashi HG[4], Raw I[1], Dias WO[1]

[1]Centro de Biotecnologia, [2]Seção de Controle Biológico, [3]Serviço de Bacteriologia - Seção de Vacinas Aeróbicas, [4]Divisão de Produção, Instituto Butantan, SP, Brazil

Introduction: The currently available pertussis vaccines are highly efficacious. However, whole cell pertussis vaccines (WCPVs) display some reactogenicity. The acellular vaccines (APVs) are less toxic, but high costs limit their use in developing countries. The Instituto Butantan developed a new technology to remove lipopolysaccharides from the WCPV using a simplified and not expensive method, which provides a WCPV with low LPS content (WCP_{Low}) and two by-products, an APV and monophosphoryl lipid A (MPLA), to be used as adjuvant. **Objectives:** The aim of this study was to evaluate immunogenicity and protection in mice conferred by the $WCPV_{low}$ and the APV obtained by pipeline production, using the MPLA as adjuvant. **Methods:** Whole cell pertussis vaccine with low rates of LPS and MPLA as by-product: the new WCP_{low} was obtained from detoxified cultures of *B. pertussis*, extracted with organic solvent. This procedure yields a significant amount of MPLA, which was used as adjuvant in pre-clinical tests of Butantan vaccines. Acellular pertussis vaccine: in the process of obtaining the WCP_{low}, the culture filtrate is a disposable product that contains the raw material for the production of an APV. It consists of a molecular ultra filtration of the detoxified pertussis culture filtrate, in 30 KDa membrane, followed by filter sterilization. **Results:** The WCP_{low} was shown to be as potent as the regular WCPV and less reactogenic, with a 5 to 10 times lower pyrogen content. The system can provide about 5% of an alternative APV, that proved to be safe, immunogenic and highly protective in mice, in the intracerebral pertussis challenge assay, after immunization with a combined diphtheria-tetanus-acellular pertussis-hepatitis B vaccine, using aluminum hydroxide or monophosphoryl-lipid A as adjuvant. **Discussion:** Our integrated process provides a pipeline production of the two pertussis vaccines, WCP_{low} and APV, and an adjuvant, MPLA, without raising the final cost of the regular WCPV production. Low cost and appropriate technologies can improve locally made vaccines and new investigations in developing countries.

Supported by FAPESP, CNPq, Fundação Butantan

2.08 Molecular cloning, expression and purification of a heat-shock protein (Cpn60) from *Bordetella pertussis*

Wolf PS[1], Lopes APY[1], Ferreira VRF[1], Sakauchi MA[2], Cainelli-Gebara VCB[1]
[1]Centro de Biotecnologia, [2]Seção de Vacinas Aeróbicas, Instituto Butantan, SP, Brazil

Introduction: *Bordetella pertussis* is a pathogenic bacterium that causes whooping cough. The current vaccine consists of whole bacterial cells with added aluminum hydroxide as adjuvant, which may cause toxic and undesirable effects. The heat-shock protein Cpn60 is a member of the chaperonin 60 family of highly conserved proteins which are involved as molecular chaperones in many essential cellular functions and additionally in cell–cell signaling. This protein is known to be one of the most immunogenic bacterial antigens and has been implicated in immune regulation at the level of innate and adaptive immunity. We have been working on the immunogenic and adjuvant properties of this protein and have proposed a simple method to obtain it from a soluble fraction extracted from the whole bacterial cells. However, in spite of a good recovery rate after the extraction and purification of Cpn60, the amount of protein effectively recovered is still low. **Objectives:** We aimed to obtain a recombinant Cpn60 in order to perform further evaluation of its adjuvant and immunogenic activities. **Methods:** The *cpn60* gene was amplified from *B. pertussis* Tohama I whole genome by PCR and cloned into pGEM-T Easy cloning vector. The gene was then linked into the expression vector pAE-6His between *Xho*I and *Hind*III restriction sites for expression in *Escherichia coli* BL21-DE3 pLysS. The culture was centrifuged, and the pellet was resuspended and disrupted with a French Press apparatus. Protein was purified by chelating chromatography on Ni^{++}-Sepharose and pooled according to SDS-PAGE analysis. The purification yielded approximately 21 mg of pure rCpn60 per liter of bacterial culture. **Results and Discussion:** We cloned the *cpn60* gene and successfully expressed and purified the recombinant protein in sufficient quantities to perform full biochemical and immunological analyses. This antigen is now being used in the immunization of mice for the evaluation of immune responses.

Supported by FAPESP, Fundação Butantan

2.09 Cloning and expression of genes that encode predicted leptospiral lipoproteins in *Escherichia coli*

Longhi MT, Oliveira, TR, Nascimento ALTO
Centro de Biotecnologia, Instituto Butantan, SP, Brazil

Introduction: Leptospirosis is an emergent global disease caused by the pathogenic bacteria *Leptospira interrogans*. Its distribution is cosmopolitan and associated with a low quality of life. Infection of animals or humans occurs from direct contact with urine or indirectly from contaminated water. The carriers may be wild or domestic animals, in particular rodents. In urban settings, most brown rats are contaminated with leptospires. Besides the adoption of prophylactic measures, new strategies such as new vaccine development is necessary to combat the disease. **Objectives**: The aim of this project was to clone two genes, LIC12895 and LIC13011, selected from the genome sequences of *L. interrogans*, to express the lipoproteins in *E. coli,* and to evaluate their reactivity with antibodies present in serum from individuals diagnosed with leptospirosis, as well as to determine their immunogenicity in mice. **Methods**: Gene sequences were obtained by PCR using complementary sequence primers and genomic DNA of *L. interrogan* serovar Copenhageni as template. The amplified genes were cloned into the expression vector pAE at *Bam*HI and *Eco*RI cloning sites. The constructions were used to transform DH5□and ampicillin-resistant clones selected for DNA mini-prep followed by analysis with the same restriction enzymes. Positive clones were sequenced in a DNA automated sequencer. The vectors containing the DNA inserts cloned at the correct reading frame were introduced into BL21 (DE3), BL21SI and BL21SI Star pLys expression *E.coli* strains. Protein expression was analyzed under different conditions, including IPTG or NaCl concentrations and induction temperatures. **Results and Discussion**: The LIC12895 and LIC13011 genes were cloned into the *E. coli* expression vector pAE without their signal peptide sequences. *E. coli* transformants were induced by the addition of IPTG or NaCl, and protein expression was analyzed by SDS-PAGE. The expected protein bands of 26.5 and 29.7 kDa, corresponding to the genes LIC12895 and LIC13011, respectively, were visualized after Coomassie blue staining. A study of whether these proteins are being expressed in their soluble or insoluble forms is currently under investigation.

Supported by FAPESP, CNPq, Fundação Butantan

2.10 Cloning, expression and characterization of new leptospirosis candidate genes uncovered from the genome as potential vaccine antigen

Hashimoto VL, Ho PL
Centro de Biotecnologia, Instituto Butantan, SP, Brazil

Introduction: Leptospirosis is a re-emergent zoonosis characterized by an acute febrile and systemic illness in humans, caused by pathogenic spirochetes belonging to the genus *Lepstospira*. The disease has global distribution but it is most common in tropical and subtropical areas. In Brazil, leptospirosis is an important economic and public health problem. The complete genomic sequence of *Leptospira interrogans* offered a new strategy for the identification of new proteins that could be vaccine candidates, since environmental control measures are difficult to implement and since there is no available vaccine for human use. **Objectives:** Secreted and surface-exposed molecules are potential targets for inducing immune responses in the host. Thus, we selected six predicted sequences coding for putative outer membrane proteins to analyze as vaccine candidates against leptospirosis and for biological characterization. **Methods:** Sequences containing signal peptide and predicted as coding for surface-exposed protein were selected from the genome of *Leptospira interrogans* serovar Copenhageni using bioinformatic tools. These sequences were cloned by PCR, and the expression of recombinant proteins was tested in three different *Escherichia coli* strains. Purification of the recombinant proteins was performed by metal affinity chromatography. Circular dichroism was performed to characterize the secondary structure. The antisera were obtained by intraperitoneal immunization of BALB/C mice. ELISA and Western blotting were carried out to confirm the titers and specificity of the antiserum. **Results and Discussion:** The six chosen sequences (LIC10291, LIC10544, LIC10881, LIC11211, LIC13435 and LIC20087) were successfully cloned, but only one recombinant protein was expressed. This recombinant protein was expressed in the soluble fraction and purified. Circular dichroism determined the secondary structure as being mainly α helix. Female mice were immunized for a period of four weeks, with booster doses at every week. ELISA revealed high titers of antiserum. Western blot analysis indicated that this protein is present only in virulent lepitospira serovar Copenhageni. Further characterizations are underway.

Supported by FAPESP, CNPq, Fundação Butantan

2.11 Cloning, expression and purification of a novel leptospiral protein with OmpA domain

Fraga TR, Nakajima E, Martins EAL
Centro de Biotecnologia, Instituto Butantan, SP, Brazil

Introduction: Leptospirosis is a serious zoonotic disease caused by infection by a highly invasive bacterium from the genus *Leptospira*. Currently, there is no vaccine against leptospirosis available for humans. In the veterinary field, a vaccine composed of heat- or formalin-killed leptospires (bacterin), which may produce an incomplete and short-term immunity, has been used. In a recent study, Loa22 (LIC10191), an OmpA-like protein was described as the first genetically defined virulence factor in *Leptospira*. Immunity is believed to be antibody-mediated; however, since leptospires are invasive microorganisms, cell-mediated response may play an important role in protection against the infection. Attenuated *Salmonella* vaccine vectors expressing heterologous antigens *in vivo* can stimulate mucosal, humoral and cell-mediated immune responses against the *Salmonella* and the recombinant antigen. **Objectives:** The aim of this work was the cloning, expression and purification of a novel OmpA-like protein, LIC10537. Additionally, the gene should be cloned for live vaccine studies using attenuated *Salmonella* expressing the heterologous antigen *in vivo*. **Methods:** The gene was amplified from genomic DNA of *L. interrogans* serovar Copenhageni by PCR, inserted into the vector pGEM-T Easy (Promega), and subcloned in the expression vector pAEsox. The constructions were confirmed by restriction analysis and DNA sequencing. Protein expression was tested in *E. coli* BL21(DE3)StarpLysS, from which the protein was purified, and in *Salmonella enterica* serovar Typhimurium SL3261, a mouse vaccine strain. **Results:** The recombinant protein LIC10537 was successfully expressed *in vitro* in both bacteria. The purification process was laborious, since the protein was expressed in the insoluble form. The protein was recovered from the inclusion bodies, refolded through a dilution protocol and purified by metal affinity chromatography followed by dialysis. **Discussion**: The genome sequence of *L. interrogans* serovar Copenhageni showed that there are five different proteins with an OmpA domain. One of the functions of OmpA is to provide physical linkage between the outer membrane and the peptidoglycan layer, which plays a critical role in the structural integrity of the bacterial cell surface. LIC10537 is a leptospiral protein with a C-terminal OmpA consensus domain that contains a peptidoglycan-associating motif. It suggests a possible role of this protein in the stabilization of the leptospiral envelope structure. With the purpose of evaluating two ways of presenting LIC10537 to the immune system, the purified protein and the recombinant vaccinal *Salmonella,* which can express the protein *in vivo,* were produced in this work. Their immunogenic properties and the types of immune response elicited are currently being tested in an animal model.

Supported by FAPESP

2.12 Searching for immunoreactive proteins of *Leptospira interrogans*

Atzingen MV[1], Abreu PAE[1], Romero EC[2], Morais ZM[3], Vasconcellos SA[3], Nascimento ALTO[1]

[1]Centro de Biotecnologia, Instituto Butantan, SP, Brazil; [2]Instituto Adolfo Lutz, SP, Brazil; [3]Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brazil

Introduction: Leptospirosis, an emerging infectious disease caused by pathogenic *Leptospira*, is a worldwide zoonosis of human and veterinary concern. Leptospiral lipopolysaccharide can elicit protective immunity, which is serovar dependent. Due to the extensive serological diversity of leptospires (~250 serovars) a search for conserved membrane proteins that may stimulate heterologous immunity is being pursued. The whole-genome sequences of *L. interrogans* serovar Copenhageni and bioinformatic tools allowed us to search for novel antigens suitable for improved vaccines and diagnostic reagents against leptospirosis. **Objectives:** We focused on the expression of three genes encoding for a predicted lipoprotein (LIC10494) and two conserved hypothetical proteins (LIC11935 and LIC12730). **Methods:** The genes were amplified by PCR from six predominant pathogenic serovars in Brazil but absent in the non-pathogenic *L. biflexa*. The DNA sequences of the chosen genes were cloned into pDEST17^{TM}, an *E. coli* vector, and the recombinant proteins were expressed in fusion with 6xHis-tag at the N-terminus, to facilitate protein purification by metal-affinity chromatography. **Results and Discussion:** Results obtained with Western blotting and ELISA methods showed that these recombinant proteins were recognized by antibodies present in human serum from a patient in the convalescent phase of the disease, suggesting that it could be useful in the diagnosis of the disease and/or a potential vaccine candidate. Further evaluation of these proteins with larger serum samples will indicate their suitability for diagnostic kit development. Evaluations of the immunoprotection of these antigens in an animal model are currently underway.

Supported by FAPESP, CNPq, Fundação Butantan

2.13 Evaluation of some hemolysins as vaccine candidates against leptospirosis

Carvalho E[1,2,3], Gonçalves AP[4], Morais ZM[4], Vasconcellos SA[4], Ho PL[1,3]
[1]Centro de Biotecnologia, [2]Laboratório de Parasitologia, Instituto Butantan, SP, Brazil; [3]Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, [4]Laboratório de Zoonoses Bacterianas, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brazil

Introduction: Leptospirosis, the disease caused by *Leptospira*, leads to high rates of mortality, especially in developing countries, where approximately 20 percent of the infected people die. The infection by *Leptospira* causes diverse damage in humans, normally a result of virulent factors produced by the bacteria. One class of important virulent factors is the hemolysins. These bacterial toxins are secreted by *Leptospira*, and cause hemolysis in the host, very often leading to severe anemia. As the iron liberated by hemolysis is essential to *Leptospira* survival, these toxins seem to be basic tools to assure the advance of the infection. Leptospirosis is considered the most disseminated zoonosis in the world, and a reemerging disease. Since there is no available vaccine for human use, several attempts to obtain a vaccine are in progress. **Objectives:** As the hemolysins are considered essential for bacterial survival and responsible for severe symptoms, we analyzed these proteins as vaccine candidates against leptospirosis. **Methods:** Recombinant *Leptospira* hemolysins were obtained and injected into hamsters. Aluminum hydroxide was used as adjuvant. Two injections were given with a 21-day interval. After 42 days from the first injection, the hamsters were challenged intraperitoneally with approximately fifty percent of the lethal dose (LD_{50}). A positive control with a commercial veterinary bacterial vaccine, and a negative control with PBS, were carried out. Hamster mortality was followed for 21 days, when the survivors were sacrificed and tested for *Leptospira* infection in the kidneys. **Results:** The four hemolysins tested here (LIC10657, LIC12631, LIC10339 and LIC13143) were not protective against leptospirosis under the conditions studied. In the group of hamsters vaccinated with one of the hemolysins there were no survivors; in each of the other three groups, we observed only three surviving hamsters (out of a total of ten). Most of the surviving animals were positive for *Leptospira* infection in the kidneys. In the positive control group (immunized with the commercial vaccine), all hamsters survived and were negative for *Leptospira* infection, while in the negative control group (vaccinated with PBS), we observed about 40% mortality with 84% of the survivors not showing *Leptospira* infection in the kidneys. **Discussion:** Although hemolysins seem to be essential for *Leptospira* survival and for leptospirosis progress, the use of these four hemolysins as a vaccine do not block the infection, maybe because there are other *Leptospira* proteins that cause hemolysis and thereby assuring the bacterial iron requirements and survival.

Supported by FAPESP, CNPq, Fundação Butantan

2.14 Evaluation of humoral immune response against the *Leptospira* antigens LIC12631 and LIC12659 as purified recombinant proteins and live recombinant attenuated *Salmonella*

Lopes LM[1], Nakajima E[1], Gonçales AP[2], Morais ZM[2], Vasconcellos SA[2], Martins EAL[1], Lopes APY[1]

[1]Centro de Biotecnologia, Instituto Butantan, SP, Brazil; [2]Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brazil

Introduction: Leptospirosis is an important worldwide distributed zoonotic disease caused by a group of highly invasive spirochetes with specificity for different mammalian hosts. It has been identified as a re-emerging infectious disease in Brazil with high costs for the public health system. Rats and other rodents are carriers of the microorganism, representing the most important sources for human infection. The *Leptospira interrogans* serovar Copenhageni is the predominant serovar causing diseases in human. Immunity conferred by lipopolysaccharide (LPS)-based vaccines does not last long and does not confer cross-protection among different serogroups. Due to the difficulties preparing multivalent LPS vaccines, the use of conserved protein antigens is an important strategy in pursuing the development of a more efficient vaccine. The analysis of the genome *L. interrogans* serovar Copenhageni allowed the investigation of new antigens. **Objective**: The aim of the study was to clone and express LIC12659 (similar to VapB–virulence associated protein) and LIC12631 (similar to Hemolysin/sphingomyelinase) to be tested as vaccine in two different forms of presentation: purified protein and live recombinant vaccine based on attenuated *Salmonella* (SL3261). **Methods:** The genes were amplified from *Leptospira* genomic DNA by PCR, cloned into the pGEMT Easy vector and then transferred to pAE expression vector and pAEsox vector (especially constructed for *in vivo* expression in *Salmonella typhimurium* SL3261). Proteins were expressed in *E. coli* Star (DE3) plysS and purified by chelating affinity chromatography on Ni^{++}-Sepharose. Recombinant proteins were analyzed by SDS-PAGE and Western blotting using antibodies raised in mice, immunized with each antigen. Hamsters were immunized with a pool of 3 antigens (also LIC10793) or with recombinant *Salmonella*-LIC12659 for challenge assay. ELISA was used for determination of antibody titers. **Results**: The genes were cloned and the recombinant proteins were expressed and purified. Two proteins were recognized in Western blotting using the sera raised against the respective antigens. The immunization of mice with isolated proteins induced very high titers (>1:500,000) of antibodies against both lepstospira antigens. Hamster immunization with the pool containing the LIC12659 or recombinant salmonella-LIC12659 resulted in similar antibody titers. The immune response observed in the hamster was lower than that observed in mice. Additional evaluations of immune response are being carried out. **Discussion:** Antigens were shown to be highly immunogenic for IgG production. The immunization of hamster with LIC 12659 showed that the recombinant *Salmonella* expressed the antigen *in vivo* and that the immune response was similar for both types of antigen presentation with a slightly increased survival in *Salmonella*-LIC12659 immunized hamsters, providing a basis to continue this approach.

Supported by FAPESP, CAPES, Fundação Butantan

2.15 MCP-1 and MIP-1α chemokines and their role in immunologic response of C3H/HeJ mice infected with *Leptospira interrogans* serovar Copenhageni

Silva JB da[1], Ramos TMV[2], Avelar KES[2], Ho PL[1], Pereira MM[2]
[1]Centro de Biotecnologia, Instituto Butantan, SP, Brazil; [2]Laboratório de Referência Nacional para Leptospirose, Fundação Instituto Oswaldo Cruz, RJ, Brazil

Introduction: Leptospirosis is a zoonotic disease caused by spirochaetes from *Leptospira* genus. Leptospiral infection causes pathologic alterations observed mainly in the lungs, liver and kidneys of infected organisms. The inflammatory chemokines and cytokines may be associated with the severity of the infection. C3H/HeJ mice are susceptible to leptospirosis, demonstrating pulmonary and renal lesions, with hemorrhagic characteristics similar to those observed in humans. **Objectives**: The aim of this work was to evaluate the role of the chemokines MCP-1 and MIP-1α in the immunity and pathogenesis of leptospirosis in C3H/HeJ mice. **Methods**: *L. interrogans* strain serovar Copenhageni isolated from a human clinical case was used throughout this study. This strain causes 100% of lethality in hamsters. Five groups of 5 C3H/HeJ mice 3 – 4 weeks old were infected (n=25) intraperitoneally with 10^6 cells, and one group was maintained as control. Mice were killed 1, 3, 5 and 6 days after inoculation and their blood, spleen, liver, kidneys and lungs were collected. Half of each organ was used for bacterial detection in mouse tissues by PCR, and the other half was used to determine MCP-1 and MIP-1α by immunoenzymatic assay (ELISA). **Results**: The results showed that the C3H/HeJ mice were susceptible to leptospiral infection. MCP-1 level was high in all the analyzed organs from the first day after inoculation, showing maximum levels in serum, liver and lungs on 5th day and in kidneys on the 6th day after inoculation. The highest levels in spleen were observed on the 3rd day followed by a decline in the subsequent days. The levels of MIP-1α were lower when compared to MCP-1 in the organs examined. Slightly increased levels were detected in the liver on the 1st day, which remained constant until the 6th day after inoculation. In kidneys, spleen and lungs, the maximum levels were detected on the 3rd day after inoculation, with a decline in the subsequent days. **Discussion:** Our preliminary results suggest an involvement of MCP-1 in leptospirosis severity and that MIP-1α is probably not directly involved in the lesions observed in the experimental model used.

Supported by IOC/FIOCRUZ, Instituto Butantan

2.16 Recombinant *Salmonella* as live vaccine against *Leptospira interrogans*

Nakajima E[1], Gonçales AP[2], Morais ZM[2], Vasconcellos SA[2], Martins EAL[1]
[1]Centro de Biotecnologia, Instituto Butantan, SP, Brazil; [2]Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brazil

Introduction: Leptospirosis is an important zoonosis caused by spirochetes of the genus *Leptospira.* In humans, the disease is associated with the rainy season in places that lack sanititation, resulting in high costs for the public health system in Brazil. About 3,600 cases were recorded last year with a 10% mortality. There is no vaccine for human use registered in the FDA (Food and Drug Administration, USA) against leptospirosis. Recently, the genome of *Leptospira interrogans* Copenhageni, the most pathogenic strain for humans, made it possible to search for vaccine candidates. Recombinant *Salmonella* is being proposed as live vaccine carrying multiple antigens. To express heterologous genes in *Salmonella* we are using an expression system based on the soxRS regulon. This system is present in many enterobacteria and responds to oxidative stress imposed by macrophages during mammalian infection. The system can be activated *in vitro* by paraquat. **Objectives:** The goal of our work was to identify Leptospira antigens, testing their potential to induce protective immunity in animals immunized with purified protein or live recombinant *Salmonella*. **Methods:** The genome of *Leptospira interrogans* serovar Copenhageni was analyzed using bioinformatic tools, looking for antigens with potential as vaccine candidates. Two systems of antigen presentation were tested: purified protein and live recombinant vaccine based on attenuated salmonella (SL3261). Six selected genes were amplified from the leptospira genome (LIC10191, LIC10793, LIC11227, LIC12302, LIC13101 and LIC10325) and cloned in vector pAE, to produce the recombinant proteins, and in vector pAEsox for *in vivo* expression, using *Salmonella* as carrier. The proteins were purified by metal affinity chromatography. **Results and Discussion:** The six genes were expressed successfully using the pAEsox vector in *E.coli* and in attenuated *Salmonella* strain SL3261, except LIC12302 and LIC13101. Two antigens (LIC10191 and LIC10793) were used to immunize mice either with purified proteins or live recombinant *Salmonella*, to investigate possible differences in the immune response related to the two forms of antigen presentation. Purified proteins induced high antibody titers, while recombinant *Salmonella* resulted in low levels of antibodies against leptospiral antigens. The antibodies were used for Western blotting and ELISA. The same antigens were used for hamster immunization followed by challenge assay for the measurement of protective capacity against *Leptospira* infections. We observed that hamsters are more susceptible to both types of immunization with higher antibody titers raised. However, these antibodies were not enough to induce protection after challenge with *Leptospira*. Comparing the protection between groups immunized with the different forms of antigen presentation, only one animal survived in the group immunized with the purified protein LIC10191 (10%), while four animals survived in the group immunized with the recombinant *Salmonella* carrying LIC10191 (44%). It is important to note that the surviving animals were infected with *Leptospira* and appeared sick by the end of the experiments.

Supported by FAPESP, CNPq, CAPES, Fundação Butantan

2.17 Proteomic comparison between *in vitro* cultivated and tissue-derived leptospires

Vieira ML[1], Pimenta DC[2], Abreu PAE[1], Vasconcellos SA[3], Morais ZM[3], Nascimento ALTO[1]

[1]Centro de Biotecnologia, [2]Laboratório de Bioquímica e Biofísica, Instituto Butantan, SP, Brazil; [3]Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brazil

Introduction: The spirochaete bacterial pathogen *Leptospira interrogans* is the causal agent of leptospirosis, a worldwide zoonosis of human and veterinary concern. To date, there are no effective vaccines available for either animal or human use. The leptospiral genome sequencing revealed several novel proteins that could be involved in pathogenesis and virulence, and may thus constitute important vaccine candidates against leptospirosis. It is known that some bacterial pathogens can modulate their protein expression profile according to the environmental conditions. During the pathogenic cycle, the leptospires are subjected to different environmental conditions that occur in mammalian hosts and water transmission, and may thus express different protein sets to adapt to each condition. Additionally, it is well documented that some leptospiral proteins involved in virulence are down-regulated after several passages of *in vitro* cultivation, losing their ability to infect. **Objectives:** Based on these assertions, we set out to investigate aspects of the pathogenesis and virulence of these bacteria by analyzing the differential protein expression between non-virulent culture-attenuated and virulent strains of *L. interrogans* serovar Pomona by proteomics. **Methods:** Hamsters were inoculated with virulent bacteria followed by cultivation of kidney- and liver-derived leptospires. The non-virulent bacteria were obtained by several culture-passage steps. The total bacterial protein extracts were separated by two-dimensional gel electrophoresis (isoelectrofocusing and SDS-PAGE). For each sample (liver, kidney and non-virulent), three independent 2D gels were run. The resulting protein spots were excised from the gels, trypsin-digested and the peptides analyzed by MALDI-TOF mass spectrometry. For the identification of the proteins, the list of the peptide m/z values obtained for each trypsin-digested spot were analyzed on the peptide mass fingerprint (PMF) mode of the MASCOT server. **Results and Discussion:** Considering all the protein extract gels studied, 1251 spots were processed, resulting in the identification of 466 spots. The spots identified represent 112 different proteins, with some of them being present in multiple spots distributed along the gels. By bioinformatics analysis it was possible to observe that the identified proteins represent all cellular compartments (inner membrane, outer membrane, periplasmic space and cytoplasm) and every major functional category of the leptospires. Interestingly, 34 hypothetical proteins were identified, confirming their expression *in vivo*. Several surface-exposed proteins and lipoproteins with unknown function were identified as being expressed in the virulent conditions, which may suggest their possible role in virulence. The data obtained in this work contribute to the understanding of leptospiral biology and highlight some proteins that may serve as vaccine constituents against leptospirosis.

Supported by FAPESP, CNPq, Fundação Butantan

2.18 Comparison of antigenicity and immunogenicity of two dynein light chain from *Schistosoma mansoni*

Diniz PP[1], Rocha MO[1], Miyasato PA[2], Kawano T[2], Martins EAL[1]
[1]Centro de Biotecnologia, [2]Laboratório de Parasitologia, Instituto Butantan, SP, Brazil

Introduction: Schistosomiasis is considered one of the most important endemic diseases in the world, with more than 200 million people infected in 76 countries, including Brazil. It is estimated that 600 million people are in the risk area. Around 10% of infected individuals can develop serious symptoms. Few drugs are available for treatment. Still, some parasites become resistant, and chemotherapy does not prevent reinfections. Many efforts are being made for a vaccine development. In the transcriptome analysis of *Schistosoma mansoni,* a family of 18 dynein light chain (DLC) homologs was identified. Two members of this paralogous family were previously described, indicating the presence of the two DLCs in the tegument of adult worm by immunomicroscopy. Considering that these proteins are exposed to the host immune system, we decided to investigate their potential as vaccine. Attenuated *Salmonella* strains are being used as live recombinant vaccine carrying heterologous genes. Many studies have shown that *Salmonella* can drive the immune response to the cellular type, which is desirable for protection against parasites. **Objectives:** Our main objective was to evaluate the antigenicity and immunoprotective capacity of two DLC from *S. mansoni*, by presenting the antigens to the mouse immune system as purified protein or carried by a recombinant *Salmonella* vaccine strain. **Methods:** Two genes of DLC were cloned in the expression vector pAEsox, and the proteins were induced and purified by metal-chelating affinity chromatography. The same genetic constructions were cloned for *in vivo* expression in attenuated *Salmonella*, expecting adjuvancy for TH1 type immune response. The different groups of animal were immunized with purified protein or recombinant *Salmonella* four times at 15-day intervals. Humoral immune response to DLCs was estimated by ELISA and Western blotting. After the fourth immunization, mice were infected with cercaria for the challenge assay. Forty-five days after the infection, the animals were sacrificed and perfused for recovery of adult worms. **Results:** The sera obtained from immunization with the purified proteins DLC1 and DLC4 showed that these proteins are highly antigenic in mice. Antibodies against DLC1 and DLC4 were not detected after immunization with recombinant *Salmonella*. The Western blotting assay for protein DLC1 confirmed that the antibodies in the sera against DLC1 recognized the purified protein. Besides, some fractions of total protein extract from adult worm were recognized by the antibody anti-DLC-1. Interestingly, in the challenge assay the group immunized with the recombinant *Salmonella* DLC1 showed a lower number of adult worms recovered after 45 days of infection. **Discussion:** The present study confirmed previous data showing that DLC1 is a highly antigenic protein. The immunoprotective capacity of this protein seems to be significant when carried by attenuated *Salmonella*. However, our results are not yet conclusive, because the number of adult worms recovered in all the experimental groups was lower than that expected. The method for challenge assays needs to be improved. The anti-DLC mouse antibodies will be used in future immunolocalization assays.

Supported by Fundap, Fapesp, Fundação Butantan

2.19 Effect of hemolymph from *Lonomia obliqua* in rabies virus glycoprotein expression in Drosophila S2 cells

Greco KN[1], Souza APB[1], Pereira CA[1], Astray RM[1], Moraes RHP[2], Mendonça RZ[1]

[1]Laboratório de Imunologia Viral, [2]Laboratório de Parasitologia, Instituto Butantan, SP, Brazil

Introduction: Protein expression systems have been intensely used for the production of recombinant proteins. However, low expression amounts have limited the industrial production of some proteins of interest. In biotechnological processes, high-density cultures are an essential factor in maximizing product yield. With this objective, numerous processes have been used. **Objectives:** In this study, the benefits of cell culture supplementation with hemolymph from *Lonomia obliqua* were investigated and the effects on recombinant rabies virus glycoprotein (GPV) production and on S2AcGPV2 cell growth were determined. **Methods:** Hemolymph was fractionated by gel filtration and ionic exchange chromatography, fractions were identified by SDS-PAGE, and the effects were characterized in cultures of S2 cells transfected with the GPV gene. All experiments were carried out in 100-ml shake flasks (working volume: 20 ml). The cultures were performed in an orbital shaker at 100 rpm and 28 ºC. To test for GPV expression, immunofluorescence and flow cytometry assays were performed to measure the % of cells expressing the GPV. The GPV concentration in the S2AcGPV2 cell cultures was estimated by ELISA. **Results:** The results obtained demonstrated that hemolymph has a beneficial effect in insect cell culture. The addition of 1% of *Lonomia obliqua* hemolymph increased the rabies virus glycoprotein expression in drosophila S2 recombinant cells up to 80%, and in the culture supplemented with hemolymph, the cells remained viable for longer periods of time and with higher cell yields, the S2AcGPV2 cell growth up to 30% when compared to controls with TC-100 medium with 10% of FBS. The hemolymph was fractionated into three pools of proteins (Pool 1, 2 and 3) and the chromatographic fraction responsible for increase the GPV synthesis was pool 1, when added at 2% to the S2AcGPV2 cultures. **Discussion:** The analyses of GPV expression showed the presence of a potent protein in *Lonomia obliqua* hemolymph. The addition of this hemolymph in S2AcGPV2 culture seems to delay cell death by an anti-apoptotic protein that can be interacting with any apoptotic pathway, and increase the recombinant GPV synthesis. The protein responsible for this effect is a protein of high molecular weight, which is present in the first chromatographic fraction, designated as pool 1.

Supported by FAPESP, CAPES, Fundação Butantan

2.20 Rabies virus glycoprotein (RVGP) expression in *Drosophila melanogaster* S2 cells co-transfected or transfected with genetic expression and selection constitutive vectors

Santos AS, Lemos MAN, Pereira CA, Jorge SAC
Laboratório de Imunologia Viral, Instituto Butantan, SP, Brazil

Introduction*:* An important feature of insect cells cultures is that they reach high cell densities with low costs and simple maintenance. In this work we used *Drosophila melanogaster* S2 cells to study the expression of a viral protein (RVGP – rabies virus). **Objective***:* The objective of this work was to compare the efficiency of co-transfection with the vectors pAcGPV + pCoHygro to transfection with only one vector pAcGPVHygro in S2 cells. **Methods:** The populations transfected S2AcGPVHygro and co-transfected S2AcGPV were analyzed for RVGP expression and compared by direct immunofluorescence, confocal microscopy, ELISA, FACS and Western blotting. **Results and Discussion**: We obtained stable cells cultures that were co-transfected with the vectors pAcRVGP (expression vector) + pCoHygro (hygromycin selection vector) and transfected with pAcRVGPHygro (expression and selection vector). The recombinant RVGP was quantified by immunoassay (ELISA), and the data showed 280.8 ng/10^7cells of RVGP expression on the pAcRVGPHygro transfected cells and 112.2 ng/10^7 cells of RVGP expression on the pAcRVGP+ pCoHygro co-transfected cells. Western blotting analysis showed a 65KDa RVGP monomeric structure. We expect to optimize the RVGP production by selecting subpopulations of S2 recombinant.

Supported by FAPESF, CNPq, Fundação Butantan

2.21 Influence of BiP secretion sequence signal on the expression or rabies virus glycoprotein (RVGP) in *Drosophila melanogaster* S2 cells

Lemos MAN, Santos AS, Astray RM, Jorge SAC, Pereira CA
Laboratório de Imunologia Viral, Instituto Butantan, SP, Brazil

Introduction: Gene expression in cells has been a powerful tool in biotechnology. Several biological products have been generated through the construction of gene vectors that upon cell transfection can be expressed and give rise to active proteins. In this work we used *Drosophila melanogaster* S2 cells to study the expression of a viral protein (RVGP) using inducible (by $CuSO_4$) gene vectors that have also a selection gene (hygromycin). In our laboratory we already have good preliminary data concerning the study of viral protein expression. However, until now we have transfected cells with vectors with the BiP secretion signal. **Objectives:** As the RVGP gene already possesses a secretion signal, we are studying an approach of cell transfection with vectors without BiP. **Methods:** We have already constructed the gene vectors (pMTRVGP and pMTRVGP-Hygro) with the viral protein gene (RVGP) and with or without the selection gene (Hygro). The pMTRVGP vector was constructed with the inserted RVGP gene under the control of drosophila metallothionein promoter into pMT/V5-HisA. The pMTRVGP-Hygro vector was constructed with the inserted selection gene under the control of drosophila Copia promoter into pMTRVGP. **Results:** S2 cells were transfected with pMTRVGP-Hygro vector and co-transfected with pMTRVGP + pCoHygro using cellfectin and were selected with hygromycin B. The selected S2MTiRVGP (Mc 4), S2MTiRVGP-H (Mc 5), S2MTRVGP (Mc 6) and S2MTRVGP-H (Mc 7) cells were cultivated, and upon induction by 700 μM of $CuSO_4$, the expression of the recombinant protein was evaluated. The RVGP was detected by immunofluorescence and ELISA. The data obtained showed an expression of 86 ng/10^7 cells of RVGP in S2MTRVGP (Mc 6) cells and 648 ng/10^7 cells of RVGP in S2MTRVGP-H (Mc 7) cells. **Discussion:** These data suggest that the signal BiP harms the expression of the GPV in the cells S2MTiRVGP (Mc 4) and S2MTiRVGP-H (Mc 5). We believe that the presence of this signal can be intervening in the formation of the protein modifying its characteristics.

Supported by FAPESP, CNPq, Fundação Butantan

2.22 Expression of rabies virus glycoprotein (RVGP) by BHK-21 cells infected with recombinant *Semliki forest* virus

Astray RM[1], Benmaamar R[2], Wagner R[2], Pereira CA[1]
[1]Laboratório de Imunologia Viral, Instituto Butantan, SP, Brazil; [2]Département Récepteurs et Protéines Membranaires, Université Louis Pasteur, France

Introduction: The production of many complex proteins for therapeutic or immunization purposes needs the completion of all the post-translational modifications that are critical for the access to antigenicity or function when the protein is tested in an *in vivo* model. Glycosylsation, palmitoylation and correct folding mediated by specific enzymes are the most common related processes involved in the generation of a good quality protein. Therefore, eukaryotic hosts are the best indicated expression systems for the production of post-translation dependent proteins, as they have the appropriate cell machinery for protein maturation. Here, we report the utilization of the *Semliki forest* virus (SFV-based) expression system to produce rabies virus glycoprotein (RVGP). The SFV system has the advantages of generating high-level expression of functional membrane proteins, of being relatively easy and safe to handle, and of being scalable. **Objectives:** The aim of the study was to evaluate the RVGP production in BHK-21 cells infected with a recombinant SFV system. **Methods:** The RVGP gene was cloned into an optimized pSFV expression vector. BHK-21 cells were cultivated and electroporated with RNAs obtained from recombinant plasmid (coding for virus replicase and RVGP) and helper plasmid (coding for viral structural proteins) in order to obtain inactivated viral particles containing the RVGP gene under the control of a SFV transcription promoter. After activation, the viral particles were used to infect new BHK-21 cultures. The RVGP expression level in cell lysates was evaluated by Western blotting utilizing anti-RVGP polyclonal antibodies. The proper RVGP conformation was accessed by immunofluorescence of fixed BHK-21 cells labelled with monoclonal fluorescein-conjugated anti-RVGP antibodies specific for the trimeric form of the protein. **Results:** The system showed good RVGP expression level at 24h and 48h after infection as determined by Western blotting. The adequate RVGP conformation was confirmed by monoclonal antibody recognition in the immunofluorescence assay. The major concentration of RVGP in infected cells seems to be localized in the plasma membrane. **Discussion:** The easy handling and the high level of protein expression of the SFV system make it a promising approach to produce complex proteins of interest. The good quantity and quality of the RVGP obtained with low cell densities make the system a candidate to a scaled-up production process prior to protein purification for antigen production. Some steps (infection and virus activation) are still the subject of standardization studies.

Supported by FAPESP, CNRS

2.23 HPV oncoprotein expression, iron endocytic pathway and mitochondria in mammalian cells

Szulczewski V[1], Aires KA[2], Bazan S[2], Porto APAGK[1], Palumbo ACM[1], Benedetto DM[1], Silva KA[1], Boccardo EM[3], Villa LL[3], Ho PL[2], Armbruster-Moraes E[1], Cianciarullo AM[1]

[1]Laboratório de Genética, [2]Centro de Biotecnologia, Instituto Butantan, SP, Brazil; [3]Laboratório de Virologia, Instituto Ludwig para a Pesquisa do Câncer, SP, Brazil

Introduction: Infection by certain human papillomaviruses (HPVs) represents the major risk factor for the anogenital cancer. The HPV genomes persist as episomal molecules in HPV-preneoplastic lesions and integrated into the host cell genome in chromosomes and mitochondrial DNA. The high-risk E6 and E7 oncoproteins drive cell proliferation through the association with PDZ domain proteins and Rb, and contribute to neoplastic progression, whereas E6-mediated p53 degradation prevents the normal repair of mutations in the cellular genome. Clathrin-mediated endocytic pathway seems to be a probable alternative entrance for HPV into the cells. **Objective:** The aim of the study was to evaluate the distribution of E6 and E7 oncoproteins, mitochondria, transferrin receptors (TfR), transferrin (Tf) and ferritin (Fe) for the iron endocytic pathway mediated by clathrin in human and animal cells, as alternative pathway for HPV infection. **Methods:** Cells transformed by HPV (HeLa, SiHa) were used as positive controls, while HPV-negative cell lines (CHO, BHK-21, 293T) were transfected with the pLXSN vector, containing the complete sequence of E7 gene (kindly donated by Dr. Denise Galloway from Fred Hutchinson Cancer Research Center, Seattle, USA), using GeneJuice® Transfection Reagent (EMD Biosciences / Merck). Primary antibodies against TfR, Tf and Fe (Dako), E6 and E7 (Oncogene) and secondary antibodies (anti-IgGs) conjugated with AlexaFluor® Dyes (Molecular Probes) were applied in immunofluorescence assays and analyzed by laser scanning confocal microscope, LSM Zeiss 510 Meta. Western blotting was used to monitor protein expression. Ultrastructural immunocytochemical assays were performed using Zeiss EM 109 transmission electron microscope. **Results:** The antibodies recognize the E6 and E7 oncoproteins in HeLa and SiHa, in the cytoplasm and nucleus, and in pLXSN vector transfected cells CHO and 293T, only in the cytoplasm. TfR were detected in abundance at the plasma membrane of cells, as well as the Fe was intensely labeled in the cytoplasm, nucleus and mitochondria. Co-localizations of E6 with mitochondria were detected in HeLa and SiHa HPV transformed cells. **Discussion:** The great amount of iron suggests a participation of this element in HPV cell transformation. The co-localization of E6 with mitochondria constitute new data, which also suggests being related to intracellular iron metabolism and probably involving the oncogenetic process of cellular transformation.

Supported by FAPESP, Instituto Butantan, Fundação Butantan, Instituto Ludwig

2.24 Production of HPV 16 L1 VLP in human cell culture for basic studies in anogenital cancer

Cianciarullo AM[1], Szulczewski V[1], Aires KA[2], Bazan S[2], Ho PL[2], Boccardo E[3], Villa LL[3], Müller M[4], Armbruster-Moraes E[1]

[1]Laboratório de Genética, [2]Centro de Biotecnologia, Instituto Butantan, SP, Brazil; [3]Laboratório de Virologia, Instituto Ludwig para Pesquisa do Câncer, SP, Brazil; [4]Tumorvirus-Specific Vaccination Strategies Group, German Cancer Research Center - DKFZ, Heidelberg, Germany

Introduction: Viral infections play a crucial role in a number of cancers, and at least 15 distinct human papillomavirus (HPV) types are described to be involved in genital, mouth, throat, and skin cancers. More than 50% of cervical cancers worldwide are attributed to HPV16. L1 is the HPV major capsid protein of approximately 55 kDa, and it can self-assemble into virus-like particles (VLPs), structurally similar to native HPV virions composed of 72 capsomers, formed by pentamers of L1 molecules. VLPs are non-infective particles applied as prophylactic vaccines, but it is possible to insert the small, plasmid, double-stranded genome DNA containing about 8,000 base pairs, converting them into infective VLPs. **Objective:** We produced virus-like particles of human papillomavirus type 16 L1 major capsid proteins (HPV16 L1 VLPs) for investigating the mechanisms by which virus infected cells cause cancer. **Methods:** Cell cultures of 293T human embryonic kidney cell line were transfected with the DNA constructs encoding for humanized L1 (L1h) antigen of HPV16, sub cloned into the mammalian expression vector pUF3L1h, using GeneJuice® Transfection Reagent (EMD Biosciences/Merck). Primary antibodies against L1, Camvir-1 (BD) and Anti-L1 HPV16 (Biodesign), and secondary antibody anti-mouse IgG_{2a} conjugated with FITC (BD) were applied in immunofluorescence assays and analyzed by laser scanning confocal microscope, LSM Zeiss 510 Meta. Western blotting was used to monitor protein expression, and ultrastructural negative staining was used for VLPs analysis with a Zeiss EM109 transmission electron microscope. Pathogen-host cell interaction assays using this HPV16 L1 VLPs are being performed. **Results:** Recombinant L1 DNA was expressed in 293T cells transfected with vector pUF3L1h in a high efficiency. At least 85% of cells expressed intracellular L1 recombinant protein and VLPs, detected by immunofluorescence with Camvir-1 and Anti-L1 HPV 16, respectively. The HPV16 L1 VLPs produced in this study interacted with non-transfected 293T cell line in immunofluorescence assays analyzed by confocal microscopy. **Discussion:** We are establishing a method for an efficient system of recombinant protein expression. The production of HPV16 L1 VLPs by transfected 293T cells opens the possibility for new basic studies concerning HPV-cell interactions and carcinogenesis mechanisms.

Supported by DKFZ-ATV, Insituto Ludwig, Fundação Butantan, Instituto Butantan

2.25 The new adjuvant nanostructured silica SBA-15: evaluation of the induced acute inflammatory response

Scaramuzzi K[1], Carvalho LV[1], Marengo EB[1], Baldon, EJ[1], Ribeiro OG[2], Sant'Anna OA[1]

[1]Laboratório de Imunoquímica, [2]Laboratório de Imunogenética, Instituto Butantan, SP, Brazil

Introduction: Adjuvants are substances used to increase the immunogenicity of antigens, as well as to accelerate and prolong effective immune response. It was demonstrated that amorphous colloidal silica shows toxic effects on macrophages and probably allows central participation of other cells, considered more efficient in the presentation of antigens (APC), such as dendritic and B cells, in the immune response development. The ordered mesoporous SBA–15 silica is a polymer that contains thick pore walls and uniform size, demonstrating high hydrothermal, thermal and mechanical stability. It shows efficacy in carrying, protecting and delivering antigens to the immune system in an efficient way, and it is also found to be non-toxic. Thus, SBA–15 can increase the immunogenicity of antigens and positively modulate the immune response of low responder individuals. **Objectives:** The aim of this work was to analyze the immunological aspects of the application of SBA-15 silica emphasizing its inflammatory response. **Methods:** In this study, mice genetically selected for high (AIR_{MAX}) and low (AIR_{MIN}) acute inflammatory response (AIR) were used. SBA-15 induced inflammatory cell recruitment was investigated in the air pouch model of subcutaneous inflammation in mice. Inflammatory parameters such as cellular influx and protein concentration of the local exudates were determined 24, 72 and 96 h after injection of 250, 500 and 2,500 μg in 0.2 ml of SBA-15 into the air pouch. Total cells in exudates were counted in a hemocytometer, and cytospins of the cells were stained with Diff-Quik (Baxter). The optical density of the proteins in the exudate was determined at 280 nm in a microplate reader (MultisKan MS reader, Labsystems, Finland). Cell subpopulations were differentially counted in cytospin preparations. The statistical analysis of the parameters studied was carried out by Student´s t-test. **Results:** Results show a low transitory inflammatory reaction induced by SBA-15. No significant differences between the two lines were observed, ascertaining that this adjuvant does not increase local inflammation. The measures of the leukocyte and serum protein accumulation in the exudates were similar in both lines. Neutrophils were observed in great number at all time periods and at all concentrations of silica tested. The injection of 2,500 μg of SBA-15 silica into the air pouch revealed that this material can significantly improve the activation of macrophages. **Discussion:** This study suggests that SBA-15 silica is a non-inflammatory material and that it is not toxic to macrophages. Moreover, SBA-15 was shown to be capable of attracting cells characteristic of adaptive immunity.

Supported by FAPESP, CNPq, Cristalia Pharmaceutics

2.26 Organ distribution and inflammation by nanostructured SBA–15 silica

Tanaka MN[1,2], Scaramuzzi K[2], Carvalho LV[2], Matos JR[3], Ribeiro OG[4], Mercuri LP[5], Sant'Anna OA[2]

[1]Universidade Cruzeiro do Sul, SP, Brazil; [2]Laboratório de Imunoquímica, [4]Laboratório de Imunogenética, Instituto Butantan, SP, Brazil; [3]Instituto de Química, Universidade de São Paulo, SP, Brazil; [5]Universidade Federal de São Paulo, SP, Brazil

Introduction: Ordered mesoporous SBA-15 silica possesses high pore volume, high surface area, and thermal and hydrothermal stability. These characteristics indicate that this material is an effective adjuvant. SBA-15 was synthesized under acid conditions by self-assembly with a triblock copolymer template, which assures its ordered structure. This material is capable of acting on the immune system, thereby improving organism defense against antigens by serving as an adjuvant. **Objectives:** The objective of this work was to investigate SBA-15 distribution in living organisms, necessary to determine the deposition of this material in different organs and to analyze its acute inflammatory response. **Methods:**Thermogravimetry/differential thermogravimetry (TG/DTG) and particle-induced X-ray emission (PIXE) were the techniques used to determine SBA-15 deposition in mouse organs (spleen, liver, lungs and kidneys). For this purpose, the low-responder (L_{IVA}) mouse line was given, by the intramuscular and oral routes, SBA-15 [100 μg in 0.2 ml of phosphate-buffered saline (PBS)], and the high-responder (H_{III}) line was not inoculated (control group). The same assays were used in mice genetically selected for high acute inflammatory response (AIR_{MAX}), inoculated by subcutaneous route and simultaneously evaluated for inflammatory reactivity. The cellular influx and protein concentration in the local exudates were determined 24, 72 and 96 h after injection of different concentrations (250 or 2,500 μg in 0.2 ml of PBS) of SBA-15 silica in an air pouch. Total cells in exudates were counted in a hemocytometer, and cytospins of the cells were stained with Diff-Quik (Baxter). **Results:** A significant concentration of SBA-15 still remained in spleen of L_{IVA} mice after 40 days and a transitory low inflammatory reaction to SBA-15 was determined after one day. Small amounts of inorganic material obtained from TG/DTG analysis did not allow silicon detection and quantification by the PIXE physical-chemical analytic technique. New studies are going to be performed on SBA-15 with oral and subcutaneous administration. Intramuscular inoculation in mice will be repeated in order to obtain the necessary material for a better analysis by PIXE. **Discussion:** This study suggests that SBA-15 silica seems to be capable of attracting cells characteristic of adaptive immunity, and as an adjuvant its deposition in spleen is highly favorable.

Supported by FAPESP, CNPq, Cristalia Laboratories

2.27 Effect of SBA-15 silica nanoparticles in the activity of bone marrow–derived macrophages from low responder mice

Carvalho LV[1], Ruiz RC[2], Scaramuzzi K[1], Marengo EB[1], Baldon EJ[1], Mercuri LP[3], Fantini MCA[4], Cabrera WH[5], Ribeiro OG[5], Tambourgi DV[1], Sant'Anna OA[1]
[1]Laboratório de Imunoquímica, [2]Laboratório de Bacteriologia, [5]Laboratório de Imunogenética, Instituto Butantan, SP, Brazil; [3]Instituto de Química, [4]Instituto de Física, Universidade de São Paulo, SP, Brazil

Introduction: Adjuvants and antigen-delivery systems promote biological interactions of relevance for antigen trafficking and activation of pathways of immunogenicity that are essential in inducing efficient immune responses. The ordered mesoporous SBA-15 silica is a polymer that due to its physical and structural properties displays adjuvant effectiveness in carrying, protecting and delivering antigen of diverse sources to the immune system. Previously reported studies have demonstrated that the role played by particulate antigen delivery is dependent on mediation of an effective uptake by antigen-presenting cells (APC). **Objectives:** The aim of this work is to evaluate the effect of SBA-15 in phagocytosis and antigen catabolism in macrophages. **Methods:** Bone marrow-derived macrophages from Swiss mice, genetically selected as low antibody responders (L_{III}; L_{IVA}) were stimulated with different concentrations of SBA-15 from 20 to 1,000 μg plus *Sacaromices cerevisae* [3 yeasts/cell] or only yeasts in control mice. After stimulation, the yeast phagocytes were determined at 1, 24, 48, 72 and 96 h after culture inoculation. **Results:** *In vitro* experiments showed an increased induction of yeast phagocytosis after macrophage stimulation with different concentrations of these nanoparticules. SBA-15 is nontoxic and does not induce cell death. Moreover, it may affect antigen catabolism of macrophages, modifying antigen processing and improving antibody responsiveness in L lines ($\mathbf{L_{III}}$; $\mathbf{L_{IVA}}$). **Discussion:** The rapid catabolism of antigens in $\mathbf{L_{IVA}}$ mice was probably the basis for the low antibody production, whereas no difference was observed at the macrophage level in the $\mathbf{L_{III}}$ mice. These results suggest that the phagocytosis of SBA-15 nanoparticles decreases the rate of immunogen catabolism, enhancing antigenic presentation and improving immune responses in low responder individuals in natural populations.

2.28 Vaccine vehicle constructed by large unilamellar vesicles sandwiched by chitosan

Rescia VC[1,2], Simões AA[1,2], da Costa MHB[1]

[1]Laboratório de Microesferas e Lipossomas, Centro de Biotecnologia, Instituto Butantan, SP, Brazil; [2]Departamento de Clínica Médica, Universidade Federal de São Paulo, SP, Brazil

Introduction: Chitosan, α- (1-4)-amino-2-deoxy-β-D-glucan, is a deacetylated chitin, a polysaccharide from crustacean shells. Its unique characteristics such as positive charge, biodegradability, biocompatibility, non-toxicity, and rigid structure make this macromolecule ideal for oral vaccine. Reverse phase evaporation vesicles (REVs) can be sandwiched by chitosan (Chi). **Objective:** The aim of the study was to evaluate the application of these particles as vehicle for diphtheria toxoid (Dtxd). **Methods:** Briefly, Dtxd in 0.5 % chitosan (in 200 µL of 10 mM acetate, pH 4.3, with or without 400 mM trehalose) was added to 60 mg of soy phosphatidylcholine and 10 mg of cholesterol dissolved in 10 ml of ethyl acetate. After micelle formation, by sonication, the organogel was formed in a rotary evaporator under vacuum (15 mm Hg/cm). The organogel was resuspended in 1% PVA. **Results:** Round chitosan-sandwiched particles (REVs-Chi) of 373 ± 17 nm containing 35.89 % Dtxd (trehalose free) or 64.23 % (with trehalose) were obtained. We observed that trehalose and lyophilization retarded the protein release during the observed time (0 - 240 min). The controlled release effect was higher after 240 min of incubation when 79 % encapsulated Dtxd (trehalose free) was released from REVs-Chi in contrast with 19% REVs–Chi containing trehalose. **Discussion:** We conclude that this new vehicle controls solute release and can be formulated for oral vaccines.

Supported by FAPESP

2.29 Mice immunized with a vaccine based on *Lactococcus lactis* expressing the variable fragment of beta intimin develop serum anti-intimin antibodies able to inhibit EPEC adhesion to HEp-2 cells

Alves PCP[1], Carbonare CB[2], Vulcano AB[1], Tino de Franco M[1], Ferreira TL[1], Santos SMR[1], Carbonare SB[1]

[1]Laboratório de Imunogenética, Instituto Butantan, SP, Brazil; [2]Laboratório de Imunologia de Mucosas, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil

Introduction and Objective: Intimin is an important virulence factor of enteropathogenic *E. coli* (EPEC), the main etiological agent of infantile diarrhea. Anti-intimin antibodies inhibit EPEC adhesion to host cells, suggesting its protective capacity. *Lactococcus lactis* is a non-pathogenic and non-invasive lactic acid bacterium, and it has been used as antigen carrier for immunization of children, elderly and immunosuppressed patients. Our vaccine is based on *L. lactis* transformed with the expression vector pMD112 cloned with the variable fragment of β intimin. The aim of this study was to evaluate the immune response evoked by this vaccine in different routes and immunization schedules and determine if the sera from these immunized mice is also able to inhibit the adhesion of EPEC to HEp-2 cells. **Methods and Results**: Groups of Balb/c mice of 4 to 6 weeks were immunized with the vaccine, the purified intimin, the control *L. lactis* or only the vehicle, in different immunization schedules and by different routes. The antigens were administered exclusively by oral (100μL/dose), subcutaneous (200μL/dose) or intranasal (20μL/dose) routes or in a combined schedule in which the subcutaneous route was followed by the oral route. We also used the adjuvant monophosphoryl lipid A (MPL) in some immunization schedules. The immune response to intimin was evaluated by ELISA in sera (IgG) and feces (SIgA). These sera were also evaluated to check their ability in inhibiting the EPEC adhesion to HEp-2 cells. Our results showed that there was induction of anti-intimin antibodies after all immunization schedules using the vaccine with 10^{10} CFU/dose, regardless of the immunization route, except for the group immunized by subcutaneous route, where the highest levels of anti-intimin IgG antibodies were observed with the use of the vaccine with 10^9 CFU/dose. There was a great individual variation in the levels of anti-intimin IgG and SIgA antibodies, which contributed to the standard deviation (S.D.) to be, most of the time, very high. We also observed that the mucosal adjuvant MPL only evoked an increase in the levels of serum antibodies when used in the short schedule by the oral route, but it did not evoke an increase in the levels of secretory antibodies, unlike what we expected. Our results also showed that the sera from the vaccinated animals were able to inhibit the adhesion of EPEC to HEp-2 cells, regardless of the antibody titers being high or low. **Discussion:** According to our results, we considered the oral route as the best immunization route for this intimin vaccine based on transformed *L. lactis*, since the oral schedules induced serum and secretory anti-intimin antibodies, which are also able to inhibit the adhesion of EPEC to HEp-2 cells. Combined schedules using subcutaneous and oral immunizations did not improve the levels of serum or secretory antibodies. Therefore, the viability of the use of this vaccine indicates the way to achieve the best immunization schedule.

Supported by FAPESP, CNPq

2.30 Wild type and $K^{409}A$ recombinant Hsp65 in Systemic Lupus Erythematosus [SLE] and the potential involvement of MHC molecules in disease progression

Marengo EB[1], Moraes LV[2], Portaro FCV[1], Mello RL[3], Carvalho LV[1], Fernandes BL[3], Faria M[1], Camargo ACM[3], Tambourgi DV[1], Rizzo LV[2], Sant'Anna OA[1]
[1]Laboratório de Imunoquímica, [3]Laboratório Especial de Toxinologia – CAT/CEPID, Instituto Butantan, SP, Brazil; [2]Laboratório de Imunologia Clínica, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil

Introduction: The Hsp60s family guide several steps during protein synthesis, being abundant in prokaryotic and eukaryotic cells and conserved during evolution. One of the major expressions of the Hsp, involved in assembly and transport of proteins, occurs in natural autoimmune processes with loss of tolerance to self antigens followed by immune-mediated injury to multiple organ systems. These proteins are expressed at low levels and have essential functions in the cell cycle and differentiation. **Objectives:** Through an original approach of inductive disequilibrium of a presumptive ordered physiological and immune conditions by homologous proteins, a hypothesis based on the physical principle of Ilya Prigogine on dissipative structures and processes, and taking into account that living systems achieve an organized and ordered state from relatively disorganized materials and new type of dynamic states, here, the putative pathological and physiological role of the wild-type (WT) and the one point mutated $K^{409}A$ recombinant Hsp65 of *M. leprae* in SLE was evaluated in vivo in genetically homogeneous [NZB x NZW]F_1 mouse model that reproduces the human disease. **Methods:** Clinical signs were analyzed, including development of ascites, pile erection, lethargy, anorexia, and the mean survival time (MST). In addition, the quantitative phenotypic IgG isotypes anti-DNA and anti-Hsp65 responsiveness were individually determined during one year of life span. **Results:** The treatment with the WT decrease the MST by 46% when compared to control ($p<0.001$) and mice presented precocious anti-DNA antibodies compared to control ones ($p<0.001$), suggesting the possible role of Hsp increasing exposure/expression of nuclear antigens. Moreover, the involvement of WT correlates with the age of administration and is dose-dependent. Groups treated with the $K^{409}A$ mutant behaved as the controls. There was macrophage induction of in vitro apoptosis/necrosis by serum from WT but not by the K409A treated hybrids. Finally, the amino acid homologies between Hsp and MHC class I or II from distinct H–2 alleles showed that the 394–418 region of $K^{409}A$ shows a higher probability and possibility of recognition by class II molecules than WT, suggesting that the mutant is recognized as non-self molecule, while the WT protein is recognized as self. **Discussion:** The involvement of the Hsp65 seems central in the establishment and progression of the SLE syndrome. Differences observed in groups treated with WT concerning earlier production of IgG1 and IgG2a anti-Hsp relative to those treated with $K^{409}A$ indicate the intervention of the immune system that could act by restoring the internal equilibrium affected by the sudden increase of Hsp molecules, since that of *M. leprae* shows homology with the mouse Hsp60. The correlation between phenotypes gave new insights into the biological role of Hsp and the significant impact of environmental factors prevailing during autoimmune processes.

Supported by FAPESP, CNPq

2.31 Bacteriostactic and fungistactic effect of phenol on sterility test validation

Agostini-Utescher CL[1], Orozco SFB[1], Mendes E[1], Suzuki ME[1], Cerda MP[1], Horton DSPQ[1], Higashi HG[2]
[1]Serviço de Controle de Qualidade, [2]Divisão Bioindustrial, Instituto Butantan, SP, Brazil

Introduction: The sterility evaluation of hyper immune sera produced at Instituto Butantan is made by filtration through membrane of 0.45 μm, washing with peptonated water and incubation in thioglycolate broth and soybean-casein medium. In order to provide Good Laboratory Practices (GLP) and assure product quality, all procedures for quality control must be validated. **Objective:** The aim of study was to determine the sensitivity of the membrane filtration technique used to determine the sterility of hyperimmune sera to validate it in the Quality Control Service Laboratory conditions. **Methods:** The phenol concentration of an antibothropic serum sample was previously determined by a spectroscopic method (0.23 g%). This sample was tested against ATCC strains *Clostridium sporogenes, Pseudomonas aeruginosa, Aspergillus niger, Candida albicans* and naturally occurring microorganisms isolated from the environment (*Bacillus* sp and *Micrococcus* sp). The sample was tested by the membrane filtration technique (0.45-μm pore size), and after washing with 0.1 % peptonated water, it was incubated for 14 days in thioglycolate broth and soybean-casein medium at 30º-35ºC and 20º-25ºC, respectively. After the incubation period the media were challenged with an inoculum (1.0 ml) containing 10 to 100 CFU/ml of each used strain. Thioglycolate broth was challenged with *C. sporogenes, P. aeruginosa* and *Bacillus* sp. while soybean-casein medium was challenged with *A. niger, C. albicans* and *Micrococcus* sp. **Results:** *C. sporogenes, P. aeruginosa* and *Bacillus* sp. showed characteristic microbial growth in thioglycolate broth medium, while *A. niger, C. albicans* and *Micrococcus* sp. were observed in soybean-casein medium after 4 days incubation at the indicated temperature. **Discussion:** The method applied to test the sterility of hyperimmune sera is effective and the phenol used in the product formulation was completely inactivated by membrane washing with peptonated water, which enables the detection of low levels of microbial contamination.

Supported by Fundação Butantan

2.32 Bactericidal/bacteriostactic effect of phenol against *Pseudomonas aeruginosa*

Agostini-Utescher CL[1], Orozco SFB[1], Mendes E[1], Suzuki ME[1], Cerda MP[1], Horton DSPQ[1], Higashi HG[2]
[1]Serviço de Controle de Qualidade, [2]Divisão Bioindustrial, Instituto Butantan, SP, Brazil

Introduction: Phenol is largely used as antimicrobial agent and it is added as a preservative in hyper immune sera production. Its bactericidal effect occurs on any bacterial protein. The effective concentration is at least 0.2 to 1.0 % and the present recommendations allow not more than 0.35 g%. As water is the most important raw material in these products and *Pseudomonas aeruginosa* is a very common microorganism in this environment, phenol concentration should contribute to assuring product quality. **Objective:** The aim of this study was to confirm the bactericidal/bacteriostactic effect of phenol against *P. aeruginosa* to determine its efficacy in product quality, which could be compromised by operational deviations. **Methods:** Phenol concentration of an antibothropic serum sample was previously determined by a spectroscopic method (0.23 g% to non-diluted and 0.115 g% to diluted). These samples were diluted and were tested against *P. aeruginosa* ATCC strain at different concentrations (10^3, 10^4, 10^5, 10^6 and 10^7). Bacterial suspensions were added to serum samples and kept under refrigeration (4-8°C) for 24h. After this incubation period, all samples were submitted to a regular sterility test, by membrane filtration, using thioglycolate broth and soybean casein medium at 30º-35ºC and 20º-25ºC respectively, for 14 days. **Results:** Against high bacterial concentrations, even 0.23 g% phenol had a non-satisfactory bactericidal/bacteriostactic effect. Phenol at 0.23 g% containing serum inhibited 80 CFU/mL, demonstrating its bactericidal/bacteriostactic effect, and phenol at 0.115 g% was sufficient to eliminate 20 CFU/mL. **Discussion:** The introduction of Good Manufacturing Practices (GMP) guidelines reduces microbial contamination during hyper immune serum production, allowing a gradual and safe reduction of phenol concentration used as a preservative.

Supported by Fundação Butantan

2.33 BacT/ALERT 3D system performance on detection of contaminants in immunobiologicals

Agostini-Utescher CL[1], Orozco SFB[1], Franzolin MR[3], Horton DSPQ[1], Higashi HG[2]

[1]Serviço de Controle de Qualidade, [2]Divisão Bioindustrial, [3]Laboratório de Bacteriologia, Instituto Butantan, SP, Brazil

Introduction: BacT/ALERT 3D (B/A) system is an automated instrument used in the early detection of bacterial and fungal contamination. It performs a non-invasive monitoring ofmicrobial metabolism through carbon dioxide production. Normally, sterility is determined by direct inoculation (DI) or membrane filtration (MF) technique, both followed by a daily visual inspection for 14 days. **Objective:** The aim of the study was to compare the B/A system performance with the standard sterility test during the operational procedures in the production of immunobiologicals, sera and vaccines, with regard to sensitivity to microbial contaminants and time required for detection. **Methods:** For the DI technique, products were directly inoculated in the suitable culture media (thioglycolate broth and soybean-casein medium). For the MF technique they were filtered through a membrane (0.45-μm pore size) which were then placed in the same above-mentioned media. BacT/ALERT SN and SA (used for anaerobic and aerobic microorganism growth, respectively) were used as well, and the products assayed were onco BCG, BCG vaccine and vaccine against influenza (VCI). When a positive flask was found, it was sent to the Laboratório de Bacteriologia of the Instituto Butantan for further microbial identification. The sensitivity was evaluated with negative (apyrogenic sterile water) and positive (ATCC and naturally occurring microorganisms) controls. After incubation time preservative containing products were challenged with an inoculum containing a known amount of ATCC and naturally occurring microorganisms. **Results:** Positive contamination for onco BCG and BCG vaccine was detected by B/A system in 11.0 to 27.4 h after incubation, and with the traditional method (daily visual inspection) in 48 h to 11 days. BacT/ALERT SN and SA culture media detected an inoculum of 10 to 100 CFU/ml for microorganisms indicated in national and international guidelines for naturally occurring microorganisms. Recuperation time of microorganisms (ATCC and naturally occurring) in BacT/ALERT SN and BacT/ALERT SA culture media, previously inoculated with VCI, varied from 11.0 to 31.6 h, and 11.6 to 62.3 h, respectively. **Discussion:** The results show that the B/A system can be considered an important auxiliary analytical tool to oversee immunobiological sterility significantly reducing the product retention time during its production. Further studies should be performed in order to evaluate individual characteristics and the consistency of microorganism detection by the B/A 3D system.

Supported by Instituto Butantan

2.34 Biofilm formation by strains isolated in environmental monitoring of clean rooms

Culler HF[1], Yassui ASA[1], Mota CM[1], Agostini-Utescher CL[2], Horton DSPQ[2], Franzolin MR[1]

[1]Laboratório de Bacteriologia, [2]Serviço de Controle de Qualidade, Instituto Butantan, SP, Brazil

Introduction: Biofilm is a microbially derived sessile community characterized by cells that are attached to a substratum or interface or to each other, and are embedded in a matrix of extracellular polymeric substance produced by them. The nature of the biofilm structure and the physiological attributes of the organisms involved confer an inherent resistance to antimicrobial agents, such as antibiotics, disinfectants or germicides. Bacterial biofilms are a persistent problem in many industrial processes in the pharmaceutical industry. **Objective**: In the present work, our goal was to investigate the biofilm formation by strains isolated in environmental monitoring of clean rooms on abiotic surfaces (polystyrene). **Methods**: The 22 strains were isolated from classified environments of Instituto Butantan, through air sampling obtained by M-Air T sample using the active sampling method Split to Agar. The bacteria were identified by biochemical tests. The biofilm formation was investigated after 24 h of incubation in 96-well microtiter plates, using a colorimetric assay with crystal violet measured at 595 nm. **Results:** The bacterial species isolated were Gram-positive cocci (13 strains – 6 *Bacillus* sp., 4 *Staphylococcus aureus* and 3 *Micrococcus* sp) and non-fermentative Gram-negative bacilli (9 strains – 4 *Pseudomonas fluorescens*, 2 *Burkholderia cepacea*, 1 *Acinetobacter baumanii*, 1 *Comamonas acidovorans*, and 1 *Stenothophomonas maltophilia*). The strains studied were capable of producing biofilm on polystyrene. Ten strains showed intermediary biofilm formation (OD with values between 0.100 and 0.600 at 595 nm) and eight strains showed a superior potential (OD between 1.000 and 3.000 – mainly the *Pseudomonas fluorescens*). Only four strains (all Gram- positive cocci) displayed low biofilm production (OD < 0.100). **Discussion:** The results obtained here show the importance of determining the microorganisms present in classified environments involved in the production of biological products, as well as the awareness of their capacity for biofilm formation, reinforcing the relevance of the application of efficient control measures for these microorganisms.

Supported by Instituto Butantan

2.35 Effluent treatments from the production line of recombinant hepatitis B vaccine

Levenhagen C[1], Botosso VF[1], Oliveira DCA[1], Gouvea MN[1], Bimbo A[1], Tenório ECN[2], Higashi HG[3], Miyaki C[1]

[1]Seção de Hepatite, [2]Serviço de Virologia, [3]Divisão Bioindustrial, Instituto Butantan, SP, Brazil

Introduction: The recombinant hepatitis B vaccine produced by the Butantan Institute has been part of the National Immunization Program since 2002, while vaccine production itself was initiated in 1998. The production process consists of a fermentation step of the recombinant *Hansenula polimorpha*, cellular disintegration, and the subsequent purification of the surface antigen from the hepatitis B virus (HBsAg). The residues of the genetically modified organism (GMO) from the production process are treated with moist heat sterilization and with sodium hydroxide. With the approval of the Biosafety Law in 2005, derivatives of GMOs (recombinant proteins) must also be eliminated before being released to the environment. **Objectives:** The present study sought to evaluate the treatments of effluent by-product from the production of the hepatitis B vaccine in order to adjust these to the Biosafety norms. **Methods:** Material from all stages of the vaccine production process were decontaminated/denatured by: i) moist heat sterilization in an autoclave at 121.5 °C for 40 min, or in a reactor at 100 °C for 60 min; or ii) chemical treatment, with 0.5 N or 1.0 N sodium hydroxide (NaOH), with varying incubation times of 60 min, 90 min, 2 h, 4 h, or overnight. The efficiency of the methods were evaluated by microbiological tests (viability of the GMO- *Hansenula polymorpha*) and SDS-PAGE, Western blotting, as well as the passive hemagglutination assay (denaturing of the recombinant protein - HBsAg). **Results and Discussion:** The elimination of the inoculate from the effluents generated in the fermentation process is done using steam, and this treatment has been used with success since 2003. The HBsAg present in the by-products from the entire production chain was effectively denatured by autoclaving and in the decontamination procedure of the reactor vessel. The efficiency of the NaOH treatment has been examined for use in those situations in which steam cannot be employed. Protein denaturation was complete after incubation overnight in 0.5 N and 1.0 N NaOH. At the higher concentration, 4 h of treatment were sufficient. The residues of five vaccine lots have already been analyzed, and the results have been satisfactory.

Supported by the Fundação Butantan

2.36 Culture of unencapsulated *Streptococcus pneumoniae* strain in bioreactor

Liberman C, Silva APG, Takagi M, Sbrogio-Almeida ME, Dias WO, Leite LCC, Gonçalves VM
Laboratório de Desenvolvimento de Processos, Instituto Butantan, SP, Brazil

Introduction: According to World Health Organization, ten million deaths worldwide caused by *S. pneumoniae* are reported annually. The pathogen affects the human respiratory tract causing otitis media, sinusitis, pneumonia, meningitis and sepsis. The most common groups affected by these events are children and the elderly. Although *S. pneumoniae* has 90 serotypes, which vary by region, some vaccines had been conceived and licensed, anchored in present prevalent capsular polysaccharide serotypes of the world. One of them aggregates 23 polysaccharides from prevalent serotypes, which has questionable efficacy in protecting young children. The other one, composed of seven serotypes, therefore a multivalent vaccine too, but conjugated to protein, is costly and laborious, such as the 23-valent, to be produced in developing countries. Recently, Malley et al. suggested an inactivated whole cell vaccine of unencapsulated *S. pneumoniae*. The argument is that unencapsulated bacteria may expose all antigenic epitopes in their native configuration to the host and improve the immune response to the pathogen. The culture was originally performed in Todd–Hewitt medium, normally used for *S. pneumoniae* isolation, but not suitable for human vaccine preparation due to risk of *prion* contamination. **Objectives**: The purpose of the study was to: develop an alternative semi-defined medium, to replace the traditional one, that could be used for human vaccine production; characterize the culture conditions, such as biomass yield, optical density, growth kinetic profile; and scale it up in bioreactors maximizing the biomass. **Methods:** *S. pneumoniae* RX1Al- kan^r autolysin negative mutant, kanamycin resistant was cultured in Bacto Soytone medium in 50 ml flasks (0.5%) at 36^{o}C in 3% CO_2 and in 5L bioreactor (20%) at 36^{o}C, 0.05 L/min N_2, 0.1 bar and 150 rpm with pH controlled at 7.0. The samples were collected for OD_{600}, dry mass (weight of dry washed bacterial cells settled down in 0.22 μm membranes), pneumolysin activity, and measurement of glucose and lactate (high pressure liquid chromatography). **Results and Discussion:** The pneumolysin activity followed the bacterial growth and decreased at the stationary phase in flasks. The maximum cell density was ~250% greater in bioreactor than in flasks. All glucose was consumed in bioreactors with pH control, the lactic acid concentration reached 35g/L, where 30g/L seems to be turning point, inhibiting the bacterial growth. The dry mass concentration was 38% greater in bioreactor than in flask. Therefore, the concentration of the Soytone and the lactic acid produced in the culture seems to be critical for biomass yield of unencapsulated *S. pneumoniae*.

Supported by Pronex, FAPESP

2.37 Study of different concentrations of rotavirus (sorotypes G1 and G2) in Vero cell infection for vaccine production

Paoli RL[1], Cilli A[1], Marçal MS[1], Timenetsky MCST[2], Frazatti-Gallina NM[1]
[1]Laboratório Experimental de Rotavirus, Instituto Butantan, SP, Brazil; [2]Laboratório de Vírus Entéricos, Instituto Adolfo Lutz, SP, Brazil

Introduction: Rotavirus is one of the most common causes of gastroenteritis worldwide. The development of rotavirus vaccines and the introduction of these into global immunization programs have been high priorities for many international agencies, including WHO and the Global Alliance for Vaccines and Immunizations. A new rotavirus vaccine is under development at Instituto Butantan. It is prepared with five human-bovine reassortant rotavirus (serotypes G1, G2, G3, G4 and G9), from National Institute of Health (USA). **Objectives:** In this study we established the optimal virus concentration (MOI) to infect the Vero cells with serotypes G1 and G2. **Methods:** The Vero cell cultures in flasks were inoculated with MOI of 0.15, 0.3, 0.6, 0.9 and 1.2 and incubated at 37ºC. After 25 h, when all the cultures showed 90% of cytopathic effect, the flasks were frozen and maintained at –70ºC overnight. The flasks were thawed and the cultures were harvested. After filtration the samples were taken to determine the potency by PFU test (plate forming test). **Results:** The results obtained with the serotype G1 were 2.8×10^6, 6.2×10^6 , 7.8×10^6 , 5.4×10^6 and 1.1×10^7 PFU/ml for the cultures infected with MOI of 0.15, 0.3, 0.6, 0.9 and 1.2, respectively. The potencies of the serotype G2 were 4.6×10^5, 1.8×10^6, 1.0×10^6, 1.7×10^6 and 1.6×10^6 PFU/ml for the cells that received the 0.15, 0.3, 0.6, 0.9 and 1.2 virus concentrations, respectively. **Discussion:** The results showed that the potencies of the harvests of serotype G2 were similar to MOI of 0.3 – 1.2 and lower in the 0.15. However, in the serotype G1, the optimal MOI was 1.2.

2.38 Comparative study between two methods used to produce rotavirus suspensions in Vero cell

Paoli RL[1], Cilli A[1], Marçal MS[1], Timenetsky MCST[2], Frazatti-Gallina NM[1]
[1]Laboratório Experimental de Rotavirus, Instituto Butantan, SP, Brazil; [2]Laboratório de Vírus Entéricos, Instituto Adolfo Lutz, SP, Brazil

Introduction: The rotavirus vaccine is an important strategy to reduce the high incidence of rotavirus disease around the world. It is produced with different strains, human attenuated virus, bovine-human or rhesus-human reassortant virus. **Objectives:** In this study we evaluated the multiplication of bovine-human reassortant serotypes G1, G2, G4 and G9 in Vero cells. **Methods:** The cultures were infected with the four serotypes (MOI= 0.15) and incubated at 37ºC. When the cultures showed 90-100% cytopathic effects, 50% of the flasks of each serotype was frozen at – 70ºC. The supernatants of the remaining cultures were harvested, and the viral suspensions obtained were clarified by filtration. Samples were taken to determine the rotavirus potency by PFU (plate forming test). The cultures maintained at – 70ºC were thawed and harvested. After filtration, samples were taken for the PFU potency test. **Results:** The potency obtained in the supernatants were: 5.6×10^4, 6.8×10^4, 4.0×10^4 and 1.2×10^5 PFU/ml for G1, G2, G4 and G9 serotypes, respectively. In the frozen and thawed cultures, the results obtained found were 2.8×10^6, 4.6×10^5, 1.0×10^5 and 1.8×10^6 PFU/ml for G1, G2, G4 and G9 serotypes, respectively. **Discussion:** This data showed a significant difference in potency between the harvests of supernatants and those obtained after freezing and thawing of the cultures. Therefore, to increase rotavirus potency, harvest after freezing and thawing of the cultures is recommended.

2.39 The $CCID_{50}$ test to determine the potency of rotavirus

Paoli RL, Costa FF, Brittes AS, Frazatti-Gallina NM
Laboratório Experimental de Rotavírus, Instituto Butantan, SP, Brazil

Introduction: Rotavirus is the most common cause of severe diarrhea in children around the world. Every year, rotavirus is associated with 600,000 deaths worldwide among children younger than five years of age. The development of rotavirus vaccines and the introduction of these into global immunization programs have been high priorities for many international agencies, including WHO and the Global Alliance for Vaccines and Immunizations. The potency of this vaccine, according to WHO recommendations, is determined by three tests: $CCID_{50}$ (cell culture infection dose 50%), PFU (plate forming units test) and FFU (fluorescence forming units test). **Objectives:** The aim of the study was to evaluate two forms of virus infection in the $CCID_{50}$ test: addition of the rotavirus samples to MA-104 cells in suspension and grown as monolayers for 24 h. **Methods:** Dilutions of fourteen samples of rotavirus (G2, G3 or G9 serotypes) after activation at 37ºC for 45 min, were added to MA-104 cells in two forms: 24-h monolayers in 96-well microplates or cells in suspension. The cells were incubated with virus at 37°C for 1 h and Eagle's medium (MEM) supplemented with 1 µg/ml trypsin was added to monolayer cultures and Eagle's medium supplemented with 1 µg /ml trypsin and 2.5% calf serum to cells in suspension. The microplates were incubated at 37°C in 5% CO_2 for three days. The cultures were observed under a light microscope to determine the cytopathic effect, and the potency was calculated by the Spearman-Karber method. **Results:** The results obtained in the potency tests were: $10^{1.8}$ to $10^{4.6}$ $CCID_{50}$/ml and $10^{1.8}$ to $10^{3.8}$ $CCID_{50}$/ml when the samples of rotavirus virus were added to 24-h monolayers and in cell suspensions, respectively. **Discussion:** The results shows that the $CCID_{50}$ test were more sensitive when the samples were added to 24-h MA-104 cultures.

Supported by Fundação Butantan

2.40 Respiration rate of different insect cells cultivated in bioreactor

Pamboukian MM[1,2], Pereira CA[1], Tonso A[2]
[1]Laboratório de Imunologia Viral, Instituto Butantan, SP, Brazil; [2]Departamento de Engenharia Química, Universidade São Paulo, SP, Brazil

Introduction: Specific respiration rate (Q_{O2}) is a key variable in understanding cell metabolism and physiological state, providing useful information for process supervision and control. In this work, we cultivated different insect cells in a very controlled environment, being able to measure Q_{O2} and critical oxygen concentration (C_{crit}), i.e., the minimum dissolved oxygen concentration (DO) to keep Q_{O2} maximum, during exponential phase. Sf9 insect cells have been used for virus infection as host for foreign protein expression and bioinsecticide production. Transfected drosophila S2 cells can be used to produce different proteins. **Objectives**: The aim of this study was to determine the specific respiration of the insect cell lines studied. **Methods**: All experiments were performed in 1-liter working volume Inceltech bioreactor, maintaining temperature controlled at 28°C, agitation rate at 100 rpm, and DO at 40% air saturation, through membrane diffusion of mixed gases (O_2 and N_2) at constant k_La and total flow rate. Gibco SF900II serum free medium was used. Different cell lines were tested, as briefly detailed in results below. Q_{O2} was measured by the dynamic method, and liquid phase mass balance and C_{crit} were determined when DO reduces its decay rate without oxygen transfer. **Results and Discussion**: *Spodoptera frugiperda* (Sf9) cells (ATCC 1711) reached $10.7x10^6$ cells/mL with a maximum specific respiration rate of $7.3x10^{-17}$ mol O_2/cell.s. When infected with *Anticarsia gemmatalis* virus (AgMNPV), lower maximum cell concentration ($6.2x\ 10^6$ cells/mL), but higher Q_{O2} ($8.1x10^{-17}$ mol O_2/cell.s) was found. *Drosophila melanogaster* Schneider 2 (S2) cells (Invitrogen) achieved $51.2x10^6$ cells/mL with a maximum specific respiration rate of $3.1x10^{-18}$ mol O_2/cell.s and C_{crit} of 10% of air saturation. These cells were used in different constructions and showed the following respiration parameters: a) S2AcGPV (S2 co-transfected with rabies virus glycoprotein (pAcGPV) and pCoHygro vectors), which reached $24.9x10^6$ cells/mL: $Q_{O2} = 1.7x10^{-17}$ mol O_2/cell.s and C_{crit} = 10%; b) S2MtEGFP (S2 co-transfected with fluorescent green protein (pMtEGFP) and pCoHygro vectors), which reached $15.5x10^6$ cells/mL: $Q_{O2} = 1.9x10^{-17}$ mol O_2/cell.s and C_{crit} = 5%; c) S2AcHBsAgHy (S2 co-transfected with hepatitis B surface antigen (pAcHBsAg) and pCoHygro vectors): $Q_{O2} = 1.7x10^{-17}$ mol O_2/cell.s and C_{crit} = 15%. The transfected cells showed a higher respiration rate than the non-transfected ones, but similar C_{crit}. Concerning the Sf9 cells, S2 reached higher maximum cell concentrations and lower specific respiration rate, which can be explained by its smaller size. Further calculations are in progress, but these results present useful information for scale-up and process control of insect cells.

Supported by FAPESP and CNPq

2.41 The production of a vaccine against the hookworm *Necator americanus* in a Pilot Plant at the Instituto Butantan

Brilhante M, Ribeiro M, Sari SR, Ventura JP, Takagi M, Raw I, Ho PL, Cheng E, Gonçalves VM, Pradella JG, Martins EAL
Centro de Biotecnologia, Divisão Bioindustrial, Instituto Butantan, SP, Brazil

Introduction: Hookworms are members of the Ancylostomatoidea family. Human hookworm disease is caused by *Necator americanus*, *Ancylostoma duodenale* and *Ancylostoma ceylanicum*. The parasites infect the intestines causing bleeding, resulting in severe anemia and malnutrition, morbidity and impairment of physical and mental development. It is estimated that there are more than 500 million infected people in the world, especially in tropical and subtropical areas of developing countries. Chemotherapy is available, but it does not prevent reinfection, which occurs promptly after treatment. A consortium of investigators joined to establish work on the hookworm problem focusing on the development of a vaccine – The Human Hookworm Vaccine Initiative HHVI [(1)]. Many antigens were proposed as vaccine candidates. One of them was cloned into *Pichia pastoris* producing the protein Na-ASP-2. A method was developed for the production of recombinant protein on a laboratory scale. The Instituto Butantan was invited by HHVI to produce the first antigen for clinical trials in Brazil. **Objectives**: The aim of the study was to establish a Pilot Plant for microorganism fermentation on a scale of 60 l, as well as to proceed with the fermentation of the recombinant *Pichia pastoris* on a 60 l scale, and purification of the antigen *Na*-ASP-2 in GMP conditions for clinical trials. **Methods:** A three-phase process: 1- fermentation of the yeast consisting in a first step of feeding with glycerol and a second step of feeding and protein induction by methanol, completing 60 l volume in a BioFlo5000 fermenter. 2- cell separation using hollow fiber cartridges for microfiltration and 3- SP-Sepharose, Q-Sepharose and G25-Sephadex chromatography for protein purification. **Results**: The first Pilot Plant was set up at the Institute Butantan. The first process was performed: a working bank of cells *Pichia pastoris–Na*-ASP-2 was produced and consecutive rounds of fermentation and purification of the recombinant antigen *Na*-ASP-2 were performed. The protein was secreted into the supernatant of the yeast culture, where the average yield was 18 g per 60 l fermentation. After purification, the process provided a yield of around 72%, achieving 100% purity measured by HPLC. Documents related to the process and related to the facility "POPs" and "Prot" were developed. **Discussion**: The Pilot Plant is already set up, although more improvement is needed especially in relation to documentation. The proposed process was successfully scaled up. During the process the GMP condition was monitored, resulting in a product approved by Quality Control of the Instituto Butantan. The process was confirmed to be robust. The yield of recombinant protein was proportionally larger than with the small scale, indicating a need for further improvement.

Reference: 1. Peter J. Hotez *et al.* Int. J. Parasitol. 33:1245-1258, 2003.
Supported by Albert Sabin Vaccine Institute, Fundação Butantan, FUNDAP

2.42 Molecular characterization of tetanus toxin inactivation process

Costa GM[1], Trezena AG[2], Higashi HG[3], Sonobe MH[1]
[1]Laboratório de Bacteriologia, [2]Seção de Vacinas Anaeróbicas, [3]Divisão Bioindustrial, Instituto Butantan, SP, Brazil

Introduction: Tetanus is caused by *Clostridium tetani*, which produces the tetanus toxin responsible for all symptoms of this disease. The toxin (150 kDa) has a heavy chain of 100 kDa associated with a light chain of 50 kDa via a disulfide bond. The classical vaccines, for example DTP (diphtheria, tetanus and pertussis), require the tetanus toxin in an inactive form, as a component. The inactivation of this toxin is induced by the addition of formaldehyde and storage in an incubator (37^{o}C) for 30 days. **Objectives:** The aim of the study was to characterize the protein profiles and the macrocomplexes formed during the tetanus toxin inactivation process by acrylamide gel electrophoresis (SDS-PAGE). **Methods:** The tetanus toxin inactivation process was simulated in agreement with tetanus toxoid production conditions at Instituto Butantan. The determination of protein concentration of the collected samples was performed by the Bradford method. These samples were analyzed by acrylamide gel electrophoresis. **Results:** The concentrated tetanus toxin (cTT) showed a profile of 12 bands in the range of 139 kDa and 39 kDa. From cTT to T_{10} (period of ten minutes after formaldehyde addition) similar bands were detected. Between T_{15} and T_{40}, a complex with a molecular weight of approximately 133 kDa was predominant and 5 bands ranging from 120 kDa to 35 kDa were not detected. It was possible to observe a smear between 178 KDa and 133 KDa starting at T_{15}, which had an increased intensity over time, forming an aggregate with the described complex of 133 kDa. From T_{45} to T_{120} a complex of 100 kDa was identified, and the bands between 92 kDa and 33 kDa became undetectable. A high molecular weight complex was observed in the stacking gel starting at T_{40}. **Discussion:** The importance of the results presented here is based on the fact that the initial steps of the inactivation process are described for the first time. In comparison to literature data, the complex ranging from 178 kDa to 133 kDa is similar in molecular weight, although the parameters of harvest and the purity of the toxin are distinct between the assays. The process could be divided into 3 phases by protein profiles, one from T_0 to T_{10}, another from T_{15} to T_{40}, and a third from T_{45} to T_{120}. These results are important in the development of an important internal quality control assay for showing that vaccines have similar molecular properties and are consistently produced.

Supported by Fundação Butantan

2.43 Purification of antitetanus serum for utilization in ToBI test assay

Simões AA[1], Guilhen FB[1], Trezena AG[2], Prado SA[3], Higashi HG[4]
[1]Central de Purificação de Produtos Bacterianos, [2]Seção de Vacinas Anaeróbicas, [3]Serviço de Bacteriologia, [4]Divisão Bioindustrial, Instituto Butantan, SP, Brazil

Introduction: Toxin binding inhibition is an *in vitro* assay used in the determination of antitetanus antibodies in serum of human or animal origin. To accomplish this assay it is necessary to use antitetanus serum without albumin labeled and non-labeled with peroxidase. The removal of the albumin using the ion exchange technique is necessary because this protein may bind nonspecifically to the microplate during the assay, thereby interfering with the results. After albumin removal, the antitetanus serum was labeled with peroxidase and through the gel filtration method the enzyme-antibody complex and the non-labeled proteins were separated. All the chromatographic processes were developed by the use of the ÄKTA Explorer 100® equipment of GE Healthcare. **Objectives:** The aim of the study was to establish a reproducible method for the purification of hyperimmune antitetanus serum destined for the control of the production process. **Methods**: The antitetanus serum used in this assay was obtained from the hyperimmunization of horses according to the production procedures established in the Immunology Service of Instituto Butantan. A column of HiTrap Q FF® (GE Healthcare) negative loaded with an ion exchange resin was used to remove the albumin. The purification of antitetanus serum (without albumin) labeled with peroxidase was performed by gel filtration chromatography, using a XK16/70 column (GE Healthcare) with Sephacryl S-300 HR® (GE Healthcare) resin. After chromatography and analysis of the chromatograms, the collected samples were submitted to the determination of protein concentration by the Bradford method. All the chromatographic procedures were developed by the use of ÄKTA Explorer 100® (GE Healthcare) equipment. **Results**: According to the chromatograms, the purification procedure was appropriate and reproducible for the removal of the albumin in antitetanus serum. The use of ÄKTA Explorer enabled the ion exchange procedure to be carried out efficiently and in a short period of time (20 min). The purification of antitetanus serum (without albumin) labeled with peroxidase showed high resolution in the separation of the complex enzyme-antibody and also of the non-labeled proteins. **Discussion**: The differences in the final protein concentrations are due to the fact the initial concentration of the samples differed among themselves, as they were taken from different lots of antitetanus serum. The short period of time used during the chromatography and the reproducibility of the results are associated with the use of the equipment ÄKTA Explorer 100® equipment of GE Healthcare. The antitetanus serum purified according to the methods described in this study are being routinely used in the ToBI Test performed at Instituto Butantan.

Supported by Fundação Butantan
Acknowledgements to GE Healthcare

2.44 Characterization of atypical enteropathogenic *E. coli* (aEPEC) strains isolated in Salvador, Bahia

Abe CM[1], Blanco M[2], Dhabi G[2], Blanco JE[2], Blanco J[2], Franzolin MR[1], Taddei CR[1], Piazza RMF[1], Martinez MB[3], Elias WP[1]

[1]Laboratório de Bacteriologia, Instituto Butantan, SP, Brazil; [2]Laboratório de Referência de *E. coli*, Universidade de Santiago de Compostela, Lugo, Espanha; [3]Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, SP, Brazil

Introduction: Atypical EPEC is a term used to define EPEC strains that do not have the EAF plasmid. This EPEC category has been found in association with diarrhea in several countries, including Brazil, where currently it is the most common bacterial agent in endemic childhood diarrhea. **Objectives:** This study characterized 90 *eae*+ EAF- *stx*- fecal strains isolated from children in Bahia, Brazil. **Methods:** Standard serotyping methods, PCR assay for detecting *bfp*A and *per* markers and intimin gene sub-typing, adherence assays to HEp-2 cells and immunofluorescence (IFL) assays were performed. **Results:** Serotyping showed that 22% of the 90 strains showed non-typable O antigen. The remaining strains were distributed in 37 different serogroups, among which O111 (8%), O119 (8%), O55 (6%) and O88 (6%) were the most frequent. Among these strains, only 15 belong to 4 classical EPEC serogroups (O26, O55, O119 and O125). Strains were also distributed in 20 different H antigens, and a total of 62 different serotypes were found; however, none of them prevailed. Genes encoding 14 different types of intimin were detected: epsilon 1- (22%), beta 1- (18%), gamma 1- (12%) and theta- (10%) intimins were the most frequent. Adherence assays showed that 42% of the 90 strains did not adhere to the HEp-2 cells. The remaining strains demonstrated localized-like (LAL/27%), diffuse (DA/4%), aggregative (AA/9%), localized (LA/6%) or undetermined (UND/12%) adherence patterns. No association between adherence pattern and serotypes, serogroups or different intimin genes was observed. The presence of *bfp*A only and *bfp*A/*per* were detected in 2 and 7 strains, respectively. However, the expression of Bfp could be observed in only 5 strains (1 *bfp* and 4 *bfp*A+*per*) by IFL. **Discussion:** This study shows that *eae*+ EAF- *stx*- strains, which belong to the so-called atypical EPEC, comprise a very heterogeneous group that does not show any prevalence regarding the studied characteristics. It also corroborates the idea that typical and atypical EPEC must not be classified only by the presence of *eae* and EAF, and that other markers present in EAF plasmid as well as the BFP expression must be considered in this matter.

Supported by FAPESP, CNPq

2.45 Phenotypic and genotypic characterization of atypical enteropathogenic *Escherichia coli* (EPEC) strains belonging to classical EPEC serogroups

Bueris V[1], Ruiz RM[1], Cianciarullo AM[2], Elias WP[1]
[1]Laboratório de Bacteriologia, [2]Laboratório de Genética, Instituto Butantan, SP, Brazil

Introduction: EPEC can be classified into typical (tEPEC) and atypical (aEPEC), and the basic difference between these two pathotypes is the presence of the EPEC adherence factor (EAF) plasmid in the first group and its absence in the second. The EAF plasmid encodes the bundle-forming pilus (*bfp*) operon and the plasmid encoded regulator (*perABC*). The hallmark of EPEC infection is its ability to cause a histopathological lesion known as attaching and effacing (A/E), which results from the intimate adherence of EPEC to the intestinal epithelium. The genes necessary for the establishment of the A/E lesion are located in a pathogenicity island called LEE. In contrast to tEPEC, that displays the localized adherence pattern to cultured epithelial cells, aEPEC adhere frequently in the pattern known as localized-*like* (LAL), in which loose bacterial microcolonies can be observed on the surface of the cells only after 6 h of interaction. **Objectives:** The aim of this study was to characterize phenotypically and genotypically aEPEC strains belonging to classical EPEC serogroups. **Methods:** Fifteen aEPEC strains belonging to the serotypes O26:H11, O55:H7, O111:H9, O119:H2 and O128:H2 were analyzed for the adherence properties to HEp-2, Caco-2 and T84 cells, the ability to induce the A/E lesion in HEp-2 cells (FAS test), the presence of EPEC-associated virulence genes (*eae*, *espA*, *tir*, EAF and *bfpA*), as well as adhesin encoding genes (*efa*/*lifA*, *toxB*, *lpf*, *iha*, *paa*, *ldaH* and *saa*) by PCR, and the expression of BFP, Intimin, Tir and EspA by immunoblotting. **Results:** All strains analyzed displayed the LAL pattern after 6 h of interaction with HEp-2 cells, as well as with Caco-2 and T84 cells, and were capable of polymerizing actin at the site of the adhesion, indicating the A/E lesion occurrence also after 6 h. The presence of the main genes involved in the establishment of the A/E lesion (*eae*, *espA* and *tir*) was detected. The absence of the EAF plasmid was confirmed by the lack of detection of EAF probe sequence and *bfpA* (pilin subunit of BFP). The presence of genes associated with diarrheagenic *E. coli* adhesins was as follows: *efa/lifA* 100%, *toxB* 6.7%, *lfpA* and *iha* 33.3%, and *paa* 93.3%. Two genes, *ldaH* and *saa,* were not detected in these strains. The expression of Intimin, EspA and Tir was detected, as well the absence of BFP expression. **Discussion:** These results demonstrate that aEPEC strains belonging to the classical EPEC serogroups display the LAL pattern after 6 h of interaction with cultured epithelial cells, even in cell lines of human intestinal origin. Consequently, their ability to cause the A/E lesion also occurs after 6 h. The delay in these phenotypic characteristics may be associated with the absence of the *perABC* regulator, present only in tEPEC strains. In regard to the other adhesins, only Efa/Lif seems to be associated with the LAL pattern in these strains.

Supported by FAPESP

2.46 Functional and structural analysis of the LEE region of atypical enteropathogenic *Escherichia coli* strains that display different adherence patterns in HEp-2 cells

Rocha SP, Abe CM, Elias WP
Laboratório de Bacteriologia, Instituto Butantan, SP, Brazil

Introduction: Enteropathogenic *Escherichia coli* are classified into typical (tEPEC) and atypical (aEPEC), according to the presence or absence of the EAF plasmid, respectively. Both are capable of causing an intestinal lesion called attaching-effacing (A/E), triggered by proteins encoded by a pathogenicity island called "LEE region." The aEPEC adherence to cultured epithelial cells occurs predominantly in a localized adherence-like (LAL) pattern. However, aEPEC strains demonstrating localized (LA), aggregative (AA) and diffuse (DA) adherence patterns, as well as non-adherent (NA) strains, have also been described. **Objectives:** The main objective of this study was to analyze functionally and structurally the LEE region of aEPEC strains displaying different adherence phenotypes. **Methods:** Four representative strains showing the different adherence patterns were studied: LAL (serotype O55:H7), DA (serotype O86:H11), AA (serotype O125ac:H6) and NA (serotype O88:HNM). For the phenotypic characterization, interaction with HEp-2 and T84 cells was carried out, in addition to the detection of proteins encoded by LEE (intimin, Tir and CesT). The genotypic characterization was based on the detection of 31 genes of the LEE region employing primers based on the LEE sequence of the EPEC prototype strain E2348/69, as well as the determination of LEE insertion site in the chromosome of these strains. **Results:** The genotypic profile determined (*eae*+/EAF-/*bfpA*-/*stx*-) confirmed the classification of all strains as aEPEC. All strains expressed the proteins intimin, Tir and CesT, except for the DA strain in which intimin expression was not detected by the antiserum employed. The adherence patterns observed in HEp-2 cells were maintained in the T84 cell line. The capability of causing the A/E lesion in HEp-2 cells was detected in the LAL strain and weakly detected in the AA and DA strains. In T84 cells, only the LAL strain displayed a weak reaction. None of the strains showed the amplification of all 31 LEE genes. Three genes were not detected in the AA strain, 10 in the LAL strain, 23 in the DA strain and 14 in the NA strain. The AA and LAL strains showed LEE inserted in *selC* and the DA and NA strains in *pheV*. **Discussion:** Our results indicate that aEPEC strains, belonging to different serotypes and displaying different adherence patterns, demonstrate significant functional and structural variability of their LEE regions.

Support by FAPESP

2.47 Biofilm formation by atypical enteropathogenic *Escherichia coli* (aEPEC) strains

Culler HF, Sircili MP, Franzolin MR
Laboratório de Bacteriologia, Instituto Butantan, SP, Brazil

Introduction: Microorganisms can live and proliferate as individual cells swimming freely in the environment, or they may possess the capacity to adhere forming biofilms in close association with different types of surfaces and interfaces. This type of formation represents colonization mechanisms. Biofilms confer resistance to the action of some antibiotics in the associated bacteria and it is related to bacterial persistence. Strains of typical EPEC and their isogenic mutants in *bfpA* and *espA* form better biofilms in relation to the wild type, leading to the hypothesis that atypical EPEC strains could have a greater capacity for biofilm formation. **Objective**: The aim of this study was to determine the capacity of biofilm formation by atypical EPEC strains, on abiotic surfaces (polystyrene and glass). **Methods**: We analyzed 90 atypical EPEC strains isolated from children with acute diarrhea, using a colorimetric assay with crystal violet at wavelength of 595 nm, after 24 h incubation on coated polystyrene Costar 24-well culture dishes with or without glass coverslips, with DMEM supplemented with 0.4% glucose. The presence of the gene *espA* was determined by PCR. **Results:** The strains were capable of forming biofilm on polystyrene and glass surfaces, not showing a significant difference between the two substrates. Among the atypical EPEC strains, 79.3% showed a similar biofilm production compared toh the typical EPEC strain, whereas 22.5% showed a higher potential (DO>0.100 at 595 nm), reaching maximum DO of 1.051. The gene *espA* was found in 50.8% of the analyzed strains, being that some strains that possess *espA* had presented high production of biofilm. **Discussion:** These results raise the possibility that other structures and/or mechanisms can be involved in the pathogenesis of these strains. Adhesins not yet described can be involved in the multifactorial process of biofilm formation. The 20 strains that showed the highest capacity for biofilm formation will be determined by the direct method of counting of CFU/cm^2 adhered to biofilm after disruption with Triton X-100. Biofilm formation on substrates of HeLa epithelial cells will also be studied.

Supported by FAPESP

2.48 Intimin detection for enteropathogenic and enterohemorrhagic *Escherichia coli* diagnostic: which antibody should be used?

Menezes MA[1], Rocha LB[1], Koga PCM[1], Fernandes I[2], Magalhães CA[1], Nara JM[1], Sonobe MH[1], Elias WP[1], Piazza RMF[1]

[1]Laboratório de Bacteriologia, [2]Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil

Introduction: Adherence of enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) to enterocytes is mediated by intimin, a 94-kDa outer membrane protein that acts as an adhesin. The intimin molecule has a conserved and a variable region. The different sequences of the variable region define the intimin subtypes named after the letters of the Greek alphabet. The intimin conserved region is immunogenic and, therefore, an excellent diagnostic target. By slot-blot, colony immunoblot and immunoblot assays employing anti-intimin sera raised in rabbits against the conserved region of the protein, we detected α, β, γ, δ, ε and θ subtypes. However, this serum reacted with some unspecific proteins, even after several adsorptions with non-pathogenic *E. coli*. **Objective:** The main objective of this study was to evaluate the efficiency of different anti-intimin antibodies for detection of EPEC and EHEC isolates. **Methods:** The conserved region of purified intimin was used in rat and mouse immunizations. The popliteal lymph node cells from mice immunized with intimin were fused with SP2/O mouse myeloma cells and subcloned by limiting dilution. Dissociation constant of the monoclonal antibodies was determined by ELISA. Reactivity of the antibodies with 124 EPEC and EHEC isolates was assayed by immunoblotting analysis. **Results:** Using the immunoblot method, either the rat serum or the monoclonal antibodies reacted with a wide spectrum of intimin subtypes in different EPEC and EHEC serotypes, showing the importance of epitope linearization. Besides, rat serum demonstrated less cross-reactivity with *E. coli* proteins than did the rabbit serum. Several subclonings of the hybridomas were necessary to obtain one IgG2a and one IgG2b monoclonal antibodies, which showed dissociation constants of 1.5×10^{-8} and 1.3×10^{-8} M, respectively. **Discussion:** Concerning the polyclonal antibodies, the rat sera demonstrated better reactivity than the rabbit sera. Despite the fact that the IgG2b anti-intimin antibody showed better results in EPEC and EHEC detection, it failed in detecting some new intimin subtypes. The manipulation by site-directed mutagenesis of the single chain fragment variable (ScFv) of this antibody may improve its affinity. This will allow the large-scale production of recombinant antibodies with low cost and desirable sensitivity and specificity.

Supported by FAPESP, FINEP

2.49 Phenotypic characterization of monoclonal antibodies against Stx1 and Stx2 for detection of Shiga toxins in capture assay

Rocha LB[1], Menezes CA[1], Fernandes I[2], Piazza RMF[1]
[1]Laboratório de Bacteriologia, [2]Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil

Introduction: Shiga toxin-producing *Escherichia coli* (STEC) has emerged as a significant group of enteric pathogens worldwide, associated with a wide spectrum of human diseases, including hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), caused by the action of the potent Shiga cytotoxins (Stx1 and/or Stx2). The recent description of STEC in Brazilian cattle and the emergence of clinical events of HUS in Brazil, have spurred the need to identify them in routine diagnosis. For this reason rabbit polyclonal anti-Stx1 and anti-Stx2 antisera were raised and their efficiency evaluated by colony immunoblot, immuno dot and ELISA. Both polyclonal antisera displayed high specificity, but low sensitivity. **Objectives:** The main objective of this study was to develop and characterize Stx1 and Stx2 monoclonal antibodies. **Methods:** For monoclonal antibody (Mab) production, Balb/c mice were immunized with 10 μg of purified Stx1 or Stx2. Serum samples were obtained and subsequently analyzed by ELISA. Mice with the highest antibody titers were boosted four days prior to cell fusion, and then sacrificed by cervical dislocation. Popliteal lymph nodes were removed and fused with SP2/O mouse myeloma cells (2:1) using polyethylene glycol 4000. The fused cells were suspended and selected in HAT-RPMI medium containing 10% FBS. Hybridomas from cultures showing significant antibody production were selected by ELISA and cloned by limiting dilution culture. Also, the isotype of monoclonal antibody was determined by ELISA. The supernatants from the selected clones were filtered (0.45 μm) and purified by affinity chromatography. The dissociation constant (K_D) of the antibodies was determined by their affinity to toxin as previously described [(1)]. To further assess the reactivity of antibodies against the purified toxins, immunoblot analysis was performed using the MAbs. The neutralization potential of the antibodies was determined in the Vero cell assay. **Results:** Fusion of the myeloma cells with the popliteal lymph nodes of immunized mice with the Stx1 or Stx2 resulted in 232 and 39 hybridomas, respectively. After successive limiting dilutions, monoclonal antibodies were obtained and defined as belonging to IgG1 isotype. The dissociation constants were $3.4x10^{-10}$ M and $1.2x10^{-7}$ M for the antibodies to Sx1 and Stx2, respectively. Immunoblotting showed that the anti-Stx1 antibody recognized only the A subunit of either Stx1 or Stx2. On the other hand, the reactivity of the Stx2-antibodies was conformational; it recognized the A and B subunits of the two toxins. In the Vero cell assay, we observed that 3.1 μg/ml of the Stx1 or Stx2-antibodies inhibited the cytotoxic action of the Stx1-producing isolate. However, they were not able to inhibit completely the cytotoxic activity of the Stx2-producing isolates. **Discussion**: The data obtained in this study suggest that these monoclonal antibodies are excellent tools for immunological assays for detection of STEC isolates.

Reference: 1. Friguet B *et al.* J. Immun. Meth., 77: 305-319, 1985.
Supported by FAPESP

2.50 Cytotoxicity of plasmid-encoded toxin of EAEC-expressing strains in HEp-2 cells and inhibition of their effects by rabbit anti-Pet sera

Rossato SS, Piazza RMF
Laboratório de Bacteriologia, Instituto Butantan, SP, Brazil

Introduction: The study of bacterial toxins contributes to the comprehension of the disease pathogenesis in which they are involved and the molecular mechanisms concerning the physiological process elicited by the toxin. Enteroaggregative *E. coli* (EAEC) is one of the pathotypes from diarrheagenic *E. coli* that has an enormous epidemiological importance, and it has been considered an emerging pathogen. Recent studies have described the involvement of *plasmid encoded toxin* (Pet) in the pathogenesis of EAEC. Pet is a 108-kDa protein, member of the autotransporter class of proteins, which elicits cytotoxic effects in HEp-2 cells, characterized by elongating, rounding and detachment of the cells, and cytoskeletal changes in epithelial cells, effects dependent on serine protease activity. **Objective:** In this study, we evaluated the toxic effects of Pet EAEC-expressing strains in epithelial cells and examined the inhibition of the toxic effects by the polyclonal Pet antiserum and its IgG enriched fraction. **Methodology:** Semi-purification of Pet was carried out by ultrafiltration in a 50-kDa membrane, and cytotoxic and immunofluorescence assays were employed to visualize cytotoxic effects and their inhibition in HEp-2 cells. **Results:** In this study, 9.8 mg/ml of protein was obtained through the semi-purification of Pet, and polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate showed a polypeptide of apparent molecular weight of 108 kDa. In the cytotoxic assay of the toxin, effects were observed at a concentration of 250 μg/ml, with the presence of rounded cells and some detachment. The Pet EAEC-expressing strains caused damage to HEp-2 cells similar to that caused by semi-purified Pet. On the other hand, when culture supernatant of these isolates was added, these effects were not observed. The cytotoxicity of semi-purified Pet toxin in epithelial cells was inhibited by polyclonal Pet antiserum diluted 10 times and also the same effect was achieved when 125 μg/ml of its IgG enriched fraction was added. The PMSF (serine protease inhibitor) inhibited the toxin damage at a concentration of 1.25 μg/ml. Using the polyclonal Pet antiserum, we visualized the intracellular action of the toxin by immunofluorescence assay. **Discussion:** Pet EAEC-expressing strains caused damage to epithelial HEp-2 cells at the same magnitude as the toxic effects caused by the semi-purified Pet toxin and these effects were inhibited by polyclonal Pet antiserum and its IgG enriched fraction.

2.51 Clonal structure of atypical EPEC and the genetic relationship with other pathotypes

Bando SY[1,2], de Andrade FB[2], Trabulsi LR[2], Pestana de Castro AF[1]
[1]Instituto de Ciências Biomédicas da Universidade de São Paulo, SP, Brazil, [2]Laboratório de Bacteriologia, Instituto Butantan, SP, Brazil

Introduction: Atypical enteropathogenic *Escherichia coli* (aEPEC) is characterized by the lack of EAF (EPEC *adherence factor*) plasmid in contrast to the typical EPEC. This pathotype has been isolated from infantile diarrhea and associated with endemic diarrhea in many developing countries including Brazil where many epidemiologic studies have demonstrated that aEPEC are frequently isolated. The aEPEC is a phenotypically heterogeneous group that expresses different intimin subtypes and produces toxins characterized for other diarrheagenic *E. coli* categories such as enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC) and enterotoxigenic *E. coli* (ETEC). Little is known about the clonal structure of aEPEC and the genetic relationship with other diarrheagenic *E. coli* categories. **Objective:** This study aimed at evaluating the genetic structure of aEPEC and the relationship with other pathotypes: typical EPEC, EHEC, EAEC, DAEC and ETEC. **Methods:** A total of 22 aEPEC strains previously isolated in Bahia, Brazil were analyzed by multilocus sequencing typing (MLST). Genomic DNA was extracted using Wizard Genomic DNA Purification Kit (Promega, WI, EUA) and used as template for PCR reaction of seven housekeeping genes: *arc*A, *cya*A, *fad*D, *icd*A, *lys*P, *mtlD,* and *rpo*S. Each one of the amplicons was sequenced. ClustalX and GeneDoc were used for multiple sequence alignment, and the phylogenetic tree was constructed by a neighbor-joining algorithm based on the Jukes-Cantor model of nucleotide substitution. **Results and Discussion:** The phylogenetic tree shows two major clusters: cluster A and B. Cluster A is composed exclusively of pathogenic *E. coli*, and cluster B encompasses typical and atypical EPEC, EAEC, DAEC and EHEC. This result showed that aEPEC strains are present in all clusters of the phylogram.

Supported by FAPESP

2.52 Detection of anti-aggregation protein (dispersin) of enteroaggregative *Escherichia coli* among diarrheagenic *E. coli* pathotypes

Monteiro BT, Taddei CR, Franzolin, MR, Elias WP
Laboratório de Bacteriologia, Instituto Butantan, SP, Brazil

Introduction: Dispersin was originally described in enteroaggregative *E. coli* (EAEC) and has been characterized as a 10.2-kDa immunogenic protein encoded by the *aap* gene. *aap* is located in the EAEC high-molecular weight plasmid (pAA2), upstream to *aggR*, which encodes AggR, the transcriptional activator of EAEC virulence genes. Dispersin is secreted to the extracellular milieu where it remains attached to the surface of the bacterium, allowing its dispersal through the intestinal epithelium. We have recently isolated from an epidemiological study of the etiology of acute diarrhea in the city of Salvador (Bahia), 26 atypical enteropathogenic *E. coli* (aEPEC) strains that harbored the *aap* gene. In those strains *aggR* was not found. **Objectives:** The main objective of this study was to investigate the presence of dispersin in the other diarrheagenic *E. coli* pathotypes isolated from the same epidemiological study in the city of Salvador. **Methods:** A total of 158 strains, 113 EAEC, 38 enterotoxigenic *E. coli* (ETEC), 1 enteroinvasive *E. coli*, 1 typical EPEC, 4 Shiga toxin-producing *E. coli* and 1 enterohemorrhagic *E. coli*, were studied. A PCR reaction was standardized in order to detect simultaneously the *aap* and *aggR* gene sequences. The adherence patterns with HEp-2 cells for all dispersin positive strains were determined in the 3- and 6-h assay. **Results:** *aap* was detected in 82 EAEC (72.6%) and in 2 ETEC strains (5.2%). Among these 82 EAEC strains carrying *aap*, 67 (59.3%) also showed the *aggR* gene, and in 15 strains (13.3%) only *aap* was detected, which was the same among the *aap*-positive ETEC strains. Among the *aap*-positive EAEC strains, 92 (81.4%) displayed the aggregative adherence (AA) pattern in HEp-2 cells, 8 (9.8%) displayed diffuse adherence (DA) and 7 (8.5%) were non-adherent. The 2 *aap*-positive ETEC were also non-adherent to HEp-2 cells. **Discussion:** Our study shows that dispersin is highly prevalent in EAEC, but it is not exclusive of this pathotype, since its presence has been so far detected in atypical EPEC and ETEC. The expression of dispersin in these strains should be evaluated, since its transcriptional activator (AggR) was not detected in all strains in which *aap* was detected.

Supported by FAPESP

2.53 Prevalence of *Shigella flexneri* protein (Shf) encoding gene among enteroaggregative *Escherichia coli* strains isolated from cases of diarrhea

Vasconcellos FM[1], Taddei CR[1], Martinez MB[2], Franzolin MR[1], Elias WP[1]
[1]Laboratório de Bacteriologia, Instituto Butantan, SP, Brazil; [2]Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, SP, Brazil

Introduction: Enteroaggregative *Escherichia coli* (EAEC) is considered an emerging enteric pathogen, characterized by the aggregative adherence (AA) pattern in cultured epithelial cells. Several potential virulence factors have been described in EAEC, including adhesins, toxins and secreted proteins. These virulence markers are encoded in a high molecular weight plasmid (pAA), associated with the AA phenotype. Also, some virulence genes are located in chromosomal pathogenicity islands. Among the putative virulence markers identified during the sequencing analysis of the pAA2 plasmid, present in the EAEC prototype strain (042), is an ORF of 843 bp named *shf*. This plasmidial sequence displays homology with two ORF of *Shigella flexneri* and one of *E. coli* O157:H7 that encode the Shf protein of unknown function. This protein has 25% similarity with IcaB protein of *Staphylococcus epidermidis*, which is involved in cellular adhesion and in biofilm formation in this species. **Objectives:** Since the *shf* gene is among the most prevalent virulence factor of EAEC, as detected in previous works, the aim of the present study was to determine the prevalence of *shf* in isolates of EAEC recently isolated from cases of acute diarrhea in Brazil. **Methods:** The 113 EAEC strains employed in this study were isolated in an epidemiological study of the etiology of acute diarrhea carried out in Salvador (Bahia). These strains were characterized as EAEC by the positive reactivity with the CVD432 probe, which has been broadly used in the diagnosis of EAEC. *shf* detection was performed by PCR, employing primers based on the published *shf* sequence of strain 042 (GenBank:AF134403). The *shf* positive strains were analyzed for adherence properties with HEp-2 cells and biofilm formation on polystyrene, and these characteristics were associated with their serogroups, determined by agglutination with standard O antisera. **Results:** The *shf* sequence was detected in 41 (36%) of the 113 strains tested strains. Among the *shf*-positive EAEC strains, 31 (76%) displayed the AA pattern in HEp-2 cells and 4 (9.7%) the diffuse adherence pattern, while 6 (14.3%) did not adhere to the cells. Twenty distinct serotypes were detected, but no one predominated. The capacity to form biofilm on polystyrene was observed in the majority of the strains (80%). Some of them demonstrated higher optical densities in comparison to the prototype strain 042. **Discussion:** Our study reveals that *shf* is a prevalent virulence marker in EAEC strains recently isolated in Brazil, and that its presence is associated with the establishment of the AA pattern and biofilm formation. These data suggest that Shf plays an important role in EAEC pathogenesis.

Supported by FAPESP

2.54 Characterization of a non-adherent mutant of an atypical enteropathogenic *Escherichia coli* that displays the agregative adherence pattern in HEp-2 cells

Ruiz RM, Barros SF, Oliveira FF, Taddei CR, Uber AP, Elias WP
Laboratório de Bacteriologia, Instituto Butantan, SP, Brazil

Introduction: The *E. coli* serotype O125ac:H6 is composed of atypical EPEC strains that display the aggregative adherence pattern (AA) in HEp-2 cells, which is the main characteristic that defines enteroaggregative *E. coli* (EAEC), another pathotype of diarrheagenic *E. coli*. However, strains belonging to this serotype do not demonstrate any of the enteroaggregative *E. coli* putative virulence markers. In an attempt to identify the adhesin responsible for the establishment of the AA pattern in the O125ac:H6 serotype, a clone expressing AA was selected from a genomic library of one strain of this serotype. Sequence analysis of the insert of this clone demonstrated an ORF of 747 bp displaying homology with *ydh*F of *E. coli*. This ORF encodes a hypothetical 30 kDa-protein of unknown function. **Objectives:** Since the characterization of the adhesin responsible for the establishment of the AA pattern in the O125ac:H6 serotype is incomplete, the main objective of this study was to further characterize the genetic determinants related to the AA pattern in this serotype. **Methods:** Six *E. coli* strains belonging to the serotype O125ac:H6 and isolated from cases of acute diarrhea, two in Brazil and four in Germany, were selected for this work. The AA pattern was confirmed in HEp-2 cells employing the 6-h assay. These strains were analyzed by ribotyping employing genomic DNA digested with *Bgl*II and hybridized with the 16S and 23S probes. Mutagenesis of one selected strain (EC-292184) was performed with the transposon Tn*phoA* by conjugation. Transposon mutants were analyzed for the loss of AA in the HEp-2 cells assay. One selected non-adherent mutant and the prototype strain were also analyzed for their outer membrane protein (OMP) profiles in SDS-PAGE. **Results:** All six strains displayed the AA pattern in HEp-2 cells after 6 h of bacteria-cells interaction. The ribotyping analysis demonstrated that all strains belonged to the same clone, which permitted the selection of the EC-292184 strain as prototype of this study. The mutagenesis of this strain generated a collection of one thousand mutants. One non-adherent mutant (1::01) was selected to further analysis. The presence of the entire *ydh*F gene was detected in this mutant, suggesting that the insertion of Tn*pho*A occurred in a different gene. The adjacent regions of the transposon insertion in the mutant 1::01 were amplified by PCR. Two fragments of 1000 and 800 bp were obtained for further sequence analysis. To complement the genetic analysis, the OMP profile of the mutant was compared to the prototype strain grown at 37°C and 18°C (inhibitory temperature for the AA phenotype). This analysis revealed the absence of 21 and 41 kDa peptides in the mutant and in the prototype strain grown at 18°C. **Discussion:** The data obtained suggest that the AA pattern in HEp-2 cells displayed by the O125ac:H6 strains is multifactorial and, besides YdhF, the 21 and 41 kDa OMP identified may be involved in the establishment of this adherence phenotype.

Support by FAPESP

2.55 Experimental infection of AIRmin mice with enteropathogenic *Escherichia coli* by intragastric or intraperitoneal route

Vulcano AB, Amaral JA, Alves PCP, Carbonare CB, Tino de Franco M, Carbonare SB
Laboratório de Imunogenética, Instituto Butantan, SP, Brazil

Introduction: Enteropathogenic *E. coli* (EPEC), important agents of human diarrhea, are pathogens able to produce attaching and effacing lesions on the enterocyte membrane. The interaction between diarrheagenic *E. coli* and host cells is simulated by *in vitro* models of bacterial adhesion to cultured cells (HEp-2, Hela or CaCo), resulting in various adherence patterns. As the *in vitro* models are limited, we aimed to search for an *in vivo* model for these infections. The mice of the AIRmin strain were selected for minimal inflammatory response and showed low resistance in experimental *Salmonella* infection. **Objectives:** Our aim was to obtain an infection model by intragastric and intraperitoneal inoculations in AIR mice and to establish the DL_{50} for use in future assays to evaluate the protection of immunoglobulin preparations, as secretory human IgA and avian IgY. **Methods**: Groups of AIRmin mice 4 weeks old were infected by intraperitoneal route with doses of 10^7, 10^8 or 10^9 cfu of EPEC in 500µl. Newborn mice were infected by intragastric route with doses of 10^6, 10^7 and 10^8 cfu of EPEC in 50 µl. For the protection assays, 2 groups of 20 4-week-old mice were inoculated with the doses of 10^6, 10^7, 10^8 and 10^9 ufc of EPEC followed by treatment with 4 mg of secretory human IgA or 7 mg of avian IgY, 30 min later. In all experiments the animals were observed for 7 days for clinical evaluation and observation of numbers of deaths. After this period, we calculated the mortality rate for each group and the DL_{50} value by the Reed & Müench method. **Results:** The DL_{50} value for the newborn animals inoculated by the intragastric route was 3.2×10^6 cfu and the value for the 4-week-old mice inoculated by intraperitoneal route was 4.21×10^7. The groups of animals treated with IgA or IgY survived with greater bacterial doses. The values of the DL_{50} were 2.3×10^8 for the group treated with IgA and 1.46×10^8 for the group treated with IgY. **Discussion:** Comparing the two infection models, the DL_{50} value for the intragastric group of newborn mice was lower than the intraperitoneal group of 4-week-old mice, as expected. The intragastric route in newborn mice can be used to determine the protection conferred by maternal antibodies, acquired by transplacental transfer or colostrum antibodies acquired by breastfeeding. Passively administered IgA and IgY conferred protection to 4-week-old animals inoculated by intraperitoneal route.

Supported by FAPESP, CAPES

2.56 Atypical enteropathogenic *Escherichia coli* enterohemolysin: new phenotypic characteristics

Magalhães CA[1], Rossato SS[1], Barbosa AS[2], Piazza, RMF[1]
[1]Laboratório de Bacteriologia, [2]Centro de Biotecnologia, Instituto Butantan, SP, Brazil

Introduction: Different from typical, atypical EPEC are isolated from diarrheal outbreaks in infants, adults and animals either in developing or industrialized countries. Hemolytic toxins are produced by many $Gram^+$ and $Gram^-$ bacterial species and the lytic effect caused in cells is not exclusive to erythrocytes but also to fibroblasts, platelets, monocytes, endothelial and myocardium cells. Thus, many of these toxins are recognized as important virulence factors involved in bacterial pathogenesis. Enterohemolysin (Ehly) is expressed by different diarrheagenic *Escherichia coli* pathotypes. **Objectives:** In this work we evaluated the expression of Ehly in atypical EPEC (aEPEC) isolates and analyzed phenotypical characteristics of this toxin in different serotypes of this pathotype. **Methods:** Ninety isolates of atypical EPEC representing the main serotypes were tested for hemolytic effects on blood agar. Further we developed an ELISA assay to detect the expression of Ehly. Using this methodology, we quantified the expression of Ehly when the hemolytic isolates were grown in different culture media: tryptic soy broth (TSB), Evans medium (EM), minimal medium (MM) supplemented with lactose or glucose. Besides, the adhesion of hemolytic isolates to extracellular matrix components (ECM) as collagen I and IV, laminin, cellular and plasmatic fibronectin was investigated. **Results:** In this study the prevalence of enterohemolytic isolates was 10% on blood agar assay. The expression of EHly in two strains amongst the studied isolates, named 2062 (O171:H48) and 4192 (O111:H25) was influenced by media composition: in MM with high protein concentration no expression was observed. On the other hand, in EM Ehly was significantly expressed when the isolate 4192 was cultivated in high concentration of carbohydrate (glucose or lactose), but this expression was not observed when this isolate was cultivated in TSB even with different glucose or lactose concentrations. A significant expression of Ehly was also observed when the isolate 2062 was cultivated in EM containing 0.5% of glucose or lactose. The expression of Ehly by this isolate was not influenced by the presence of lactose in TSB growth, but in TSB containing glucose this expression was negatively modulated. Concerning adhesion to ECM all enterohemolytic isolates adhere only to cellular fibronectin matrix. **Discussion:** We conclude that despite of low prevalence of Ehly in aEPEC isolates, new phenotypical properties were described here. We observed that enterohemolytic isolates adhere to cellular fibronectin, fact that indicates that toxin molecules would be used as substrate to bacteria adhesion and even colonization. Differential Ehly expression presented in this work suggests that protein source in culture media plays a role in protein expression that was also independent of carbohydrate concentration. A virulence factor expression is really dependent on the medium in which that bacterial isolate was cultivated. Besides, a sensitive and qualitative ELISA assay was developed for detection of Ehly expression.

Supported by FAPESP

2.57 Biochemical characterization of *Salmonella* serovars and antibiotic susceptibility rates

Moda CM, Yassui ASA, Culler HF, Franzolin MR
Laboratório de Bacteriologia, Instituto Butantan, SP, Brazil

Introduction: *Salmonella* constitutes an important sanitary and economic problem in some countries in the world. These microorganisms are primary pathogens of lower animals, which are the principal source of non-typhoidal salmonellosis in humans. The transmission of *Salmonella* among humans generally occurs through the consumption of contaminated food, although person to person transmission can occur, particularly in hospitals. Members of *Salmonella* are causative agents of a variety of infections ranging from simple gastroenteritis to severe illness such as enteric fever with bacteremia. **Methods**: The *Salmonella* serovars analyzed were: *S*. Typhimurium (8), S. Agona (11), *S.* Newport (4), *S.* Enteritidis (6), *S.* Infantis (6) and *S.* Typhi (2) totalizing 34 samples which were isolated from clinical cases and from contaminated food. The *Salmonella* strains were submitted to biochemical tests such as lactose fermentation and production of hydrogen sulfide and indole. *Salmonella* strains were tested for susceptibility to 5 antibiotics with the Kirby-Bauer disc diffusion method, utilizing Müller-Hinton agar. **Results and Discussion**: All bacteria tested were negative for lactose fermentation and indole production, and they produced hydrogen sulfide, being these characteristics common to the majority of strains recovered in the literature. The strains showed 100% susceptibility to ciprofloxacin and ceftriaxone. Only one strain showed resistance to sulfametoxazol/trimethropin (5.9%) and another sample displayed resistance to cephalotin (5.9%). Only 5.9% of the strains showed intermediate sensitivity to amoxicillin/clavulanic acid and 3.0% to cephalotin. One strain of *Salmonella* Agona showed resistance to cephalotin and to sulfametoxazol/trimethropin, and intermediate sensitivity to amoxicillin/clavulanic acid. These results show the high sensitivity to five different classes of antimicrobial agents for the *Salmonella* serovars studied. It is important for laboratories to update their susceptibility profiles regularly, because individual variation of patterns may occur, and they have been unstable in recent years due to indiscriminate and constant use of antibiotics.

2.58 Influenza serologic survey in *Anseriformes* including ducks, geese and swans belonging to the public parks of São Paulo city, Brazil

Mancini DAP[1], Orico LD[2], Stroibel RC[2], Kolber M[3], Mendonça RMZ[1], Pinto JR[1]
[1]Laboratório de Virologia, Instituto Butantan, SP, Brazil; [2]Departamento de Parques e Áreas Verdes da Prefeitura da Cidade de São Paulo, SP, Brazil; [3]Faculdade de Medicina Veterinária, Universidade Metodista de São Paulo, SP, Brazil

Introduction: The literature informs that avian species, such as ducks, were the principal transmitter of the influenza type A(H2N2) to humans in 1957, which caused the pandemic disease called "Asian Flu," with high levels of morbidity and mortality around the world. Despite their important participation in the epidemiology of avian influenza, ducks are devoid of developing clinical disease. **Objective:** Taking into account this fact, this study was aimed at investigating the situation of influenza virus incidence in Brazilian *Anseriformes* flocks raised in the public parks of the city of São Paulo, SP, Brazil. **Methods:** The following city parks were sampled: Ibirapuera, Previdência, Jardim Felicidade, São Domingos and Chico Mendes. One hundred five birds including ducks- *Anas platyrhynchos* (48), geese - *Anser anser* domestic variety (29) and swans- *Cignus atratus* (28) were selected to perform the serum sample collection. This technique was carried out during the months of May to July, of the current year, 2007. The hemagglutination inhibition test (HI) was used in the serologic survey to measure hemagglutinin inhibitor antibody levels of anti-influenza virus in these avian sera. **Results:** The results of the serologic surveys showed that 20.0% of the ducks, 10.47% of the geese and 21.9% of the swans had HI antibodies (≥40 HIU/μL) against the human influenza type A (H_1N_1). Concerning the influenza virus subtype H_7N_7, the following HI antibody percentages were obtained: 29.51% for ducks; 24.76% for geese and 21.89% for swans. **Discussion:** Therefore, these data indicate that these birds must have had contact with an influenza virus on different occasions. The serologic responses were very similar among these birds, except for the geese which showed a twofold lower mean percent of antibody levels against influenza A (H_1N_1). This influenza virus transmission among birds could have occurred either via other animal species or humans in charge of the parks' animal treatment. The present study, showing the influenza serology positivity for strains of either animal or human origin, among the evaluated birds, supports the argument that there are barrier breaks between two different species. There is the possibility that the interspecies transmission, considered as a representative factor of a new and potentially pathogenic influenza strain, could emerge, provoking another flu epidemic, at anytime in our environment.

2.59 Detection and typing of Influenza Virus in children from São Paulo city, 1995 – 2005

Sacramento PR[1], Comone P[1], Durigan MS[1], Vedovello D[2], Leal AL[2], Souza TS[2], Vieira SE[3], Miyaki C[1], Tenório ECN[1], Durigon EL[2], Giglio A[3], Stewien KE[2], Botosso VF[1]

[1]Serviço de Virologia, Instituto Butantan, SP, Brazil; [2]Departamento de Microbiologia, Instituto de Ciências Biomédicas, [3]Hospital Universitário, Universidade de São Paulo, SP, Brazil

Introduction: Influenza causes frequent human epidemics associated with significant morbidity and mortality in the elderly and in those with an underlying risk factor, e.g., cardiorespiratory, renal and metabolic diseases. Epidemiologic studies have suggested that pediatric influenza can play a more important clinical role than previously thought, with an substantial socio-economic burden for the patient's family. **Objectives:** The aim of this study was to identify the prevalent types of human *Influenzavirus* in the city of São Paulo. **Methods:** We analyzed the occurrence of *Influenzavirus* in 2,007 samples of nasopharyngeal aspirates, obtained from children under 5 years old, diagnosed with respiratory illness at the University Hospital of University of São Paulo, from 1995 to 2005. The detection of viruses Influenza A and B (IA and IB, respectively) was performed by indirect immunofluorescence assay (IFA) and duplex-RT-PCR using specific primers for NS1 gene. **Results and Discussion:** One hundred and ten positive samples were detected by IFA and duplex-RT-PCR. Among these, 85.45% were positive for *Influenzavirus* A and 14.55% for *Influenzavirus* B. We observed the circulation of both types A and B in 5 years of the 11 years studied (2000 to 2002 and 2004 to 2005). The incidence of influenza in the studied population is low, but other studies have shown that infected children are important disseminators of the virus. Therefore, inclusion of this population is crucial for epidemiological studies.

Supported by FAPESP, CAPES

2.60 Ability of human colostrum and milk to neutralize SA-11 rotavirus

Santos SMR[1], Ferreira TL[1], Vulcano A[1], Alves PCP[1], Carbonare CB[2], Carbonare SB[1], Tino de Franco M[1]

[1]Laboratório de Imunogenética, Instituto Butantan, SP, Brazil; [2]Laboratório de Imunologia de Mucosas, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil

Introduction: Rotavirus gastroenteritis still remains one of the major causes of morbidity and mortality among infants and young children in both developed and developing countries. Studies have suggested that anti-rotavirus antibodies in human milk and colostrum are important for protection against this illness. However, there is no consensus about the protective effect of breast-feeding against rotavirus infection. In addition, some authors reported that breast-feeding interferes with the immune response induced by oral vaccination with attenuated viral strains. **Objective:** Our aim was to analyze the ability of human colostrum and milk to neutralize the SA-11 rotavirus strain. **Methods:** SA-11 rotavirus serotypes were cultivated and titrated in MA-104 cells. Serial dilutions of colostrums and milk samples were incubated with SA-11 10 TCID50. After 30 min incubation, the mixtures were layered over a monolayer of MA-104 cells in 96-well plates. The effect was evaluated after 48 h. The neutralization titer was determined as the reciprocal of the dilution corresponding to 60% or more of inhibited cytopathic effect. For immunoblotting (IB) assays, rotavirus proteins were transferred from gels to a nitrocellulose membrane by electrophoresis and then reacted with colostrum and milk samples. The assays were reveled with alkaline phosphatase-conjugated anti-human IgA. **Results:** We obtained a range of neutralization titers indicating some ability of colostrum and milk samples to neutralize SA-11 rotavirus. However, we could not establish any correlation between the ELISA anti-rotavirus antibody titers (obtained previously) and these neutralization titers. The IB assays were not conclusive, but they reveal a pattern of anti-rotavirus protein reaction. **Discussion:** The observed neutralization capacity of colostrum and milk was probably due to non-specific bioactive components present in them. IB assays need to be improved because the inconclusive results may have been due to technical problems.

Supported by FAPESP

2.61 Study of antifungical activity of secondary metabolites produced by microorganisms

Firoozmand LM[1], Neto CM[1], Silva JL[2], Melo IS[2], De Souza AO[1]
[1]Laboratório de Bioquímica e Biofísica, Instituto Butantan, SP, Brazil, [2]Embrapa, Jaguariúna, SP, Brazil

Introduction: Fungal infections represent a world health problem mainly in immune-compromised patients. The current antifungal drugs, in a general way, demonstrate a reduced activity spectrum against fungi and low efficacy. An intensive research for new and effective antifungal agents is needed. Many microorganisms have the ability to produce antimicrobial substances and are sources of secondary bioactive metabolites. **Objectives:** The aim of the present study was to examine the antifungal activity of secondary metabolites produced by microorganisms isolated from soil and mangrove. **Methods:** At least 40 species of non-pathogenic microorganisms were tested for antifungal activity against human pathogens by an antagonism test and the inhibition areas were measured. Some microorganisms were selected and those that showed better antifungal activity were cultivated in 200 ml of potato dextrose medium at 32ºC and shaking at 180 rpm for 1 week. Culture suspensions were centrifuged and organic extracts were obtained after extraction with hexane, dichloromethane and acetyl acetate. Minimum inhibitory concentrations (MICs) were obtained for the crude organic extracts at concentrations from 250 to 1000 μg/ml against pathogenic microorganisms such as *Trichophyton rubrum, Candida albicans, Aspergillus fumigatus and Cruptococcus neoformans.* **Results:** From the 40 species of microorganisms studied by the antagonism test, 18 formed inhibition areas with a size ≥ 2 mm. The organic extracts of these microorganisms were additionally tested using MIC test and around 55.6% showed MICs ≤ 500 μg/ml. *C. neoformans* was the more susceptible pathogen to these samples. **Discussion:** The results indicate that the microorganisms tested are a potential source of antifungal agents. These results are preliminary, and in the next step the most effective crude extracts will be purified (guided by bioassays) to isolate the effective secondary metabolite.

Supported by Fapesp

2.62 Engineering liposome to encapsulate bee venom

Silva TC[1,2], Moura SP[1], Soares de Araújo P[3], Bueno da Costa MH[1]
[1]Laboratório de Microesferas e Lipossomas, Instituto Butantan, SP, Brazil; [2]Departamento de Clínica Médica, Universidade Federal de São Paulo, SP, Brazil; [3]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, SP, Brazil

Introduction: Liposomes increase the therapeutic index and decrease toxicity and side effects of encapsulated drugs. In addition, in vaccine formulations they have been used as adjuvants. Venom immunotherapy could profit from bee venom encapsulation within liposomes. However, bee venom mellitin and phospholipase destroy phospholipid membranes. **Objectives:** Our central idea was to inhibit these enzyme activities to make possible their encapsulation. Strong indications that this formulation will be immunogenic and non-toxic are available. **Methods:** We characterized the modified bee venom (with pbb - para-bromo-phenacylbromide and/or NBS - N-bromosuccinimide) interaction with liposomes. The enzymatic activities were measured indirectly by changes in turbidity at 400 nm and by the rhodamine leak out of liposomes containing this marker. **Results:** Liposomal membranes containing pbb as a component were shown to be protected from aggregation and fusion. Membranes containing pbb maintained the same turbidity value (100%) even after incubation with modified venom, and venom hemolytic activity was completely inhibited after venom modification with pbb and NBS. In contrast, pbb-free membranes showed a 15% decrease in size. This size decrease taken together with a 50% rhodamine leak was a good indication of membrane degradation. **Discussion**: The use of modified bee venom within liposomes shows good perspectives for an increased efficacy in venom immunotherapy.

Supported by FAPESP, CAPES

3. Genetics and Immuno-regulation

3.01 Leukocyte recruitment and the airway hyperesponsiveness in the lung allergic inflammations are inhibited by PAS-1 (an immunomodulatory protein from *Ascaris suum*) due to IFN-γ- and IL-10-dependent mechanisms

Araujo CAA[1], Perini A[2], Martins MA[2], Macedo-Soares MF[1]
[1]Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil; [2]Laboratório de Terapêutica Experimental, Faculdade de Medicina, Universidade de São Paulo, SP, Brazil

Introduction: Previously, we have demonstrated that PAS-1, a protein from *Ascaris suum*, stimulates the secretion of IL-12, IFN-γ and IL-10, inhibiting lung allergic inflammation. These data indicate that PAS-1 down-modulates the allergic inflammation by cytokine-dependent mechanisms. **Objectives:** In this work, we investigated the role of IL-12, IFN-γ and IL-10 on the immunomodulatory effect of PAS-1 on leukocyte recruitment and AHR induced by ovalbumin (OVA). **Methods:** Wild type, IL-12$^{-/-}$, IFN-γ$^{-/-}$ and IL-10$^{-/-}$ C57BL/6 mice were immunized on days 0 and 7 by intraperitoneal route with OVA (50 μg) or PAS-1 (300 μg) or OVA (50 μg) + PAS-1 (300 μg) plus $Al(OH)_3$ (7.5 mg). On days 14 and 21, they were challenged by intranasal route with OVA (50 μg) or PAS-1 (300 μg) or OVA (50 μg) + PAS-1 (300 μg). On day 23, we determined the number of cells, including macrophages, neutrophils, lymphocytes and eosinophils, in bronchoalveolar lavage (BAL) and the mechanical parameters (resistance and compliance), during dose response curve to methacholine. Statistical analyses were performed by Tukey´s test. **Results:** Our results demonstrated that OVA + PAS-1-immunized wild type and IL-12$^{-/-}$ mice showed a decrease in cell number and increase in resistance in comparison, respectively, to OVA-immunized wild type and IL-12$^{-/-}$ mice. On the other hand, in OVA + PAS-1-immunized IFN-γ$^{-/-}$ and IL-10$^{-/-}$ mice the number of cells and resistance was not significantly different in relation to OVA-immunized IFN-γ$^{-/-}$ and IL-10$^{-/-}$ mice. Regarding compliance, OVA + PAS-1-immunized wild type or IL-12$^{-/-}$or IFN-γ$^{-/-}$ or IL-10$^{-/-}$ C57BL/6 mice did not showe an increase in compliance in relation to OVA-immunized mice. **Discussion:** These data demonstrated that PAS-1 did not suppress the cellular influx into airways or increase the resistance to air flow in IFN-γ$^{-/-}$ and IL-10$^{-/-}$ mice, indicating the role of IFN-γ and IL-10 on the immunomodulatory effect of PAS-1. However, PAS-1 down-modulated the cellular influx and resistance in IL-12$^{-/-}$ mice, showing that IL-12 is not important in mediating the modulatory effect of PAS-1 in lung inflammation. Taken together, these results demonstrated that PAS-1 is able to inhibit leukocyte influx and AHR (increased resistance to air flow) by IFN-γ- and IL-10-dependent mechanisms.

Supported by FAPESP

3.02 Global mRNA expression of bone marrow cells from mice phenotype-selected for maximal or minimal acute inflammation

Carneiro PS, Peters LC, Vorraro F, Borrego A, Ribeiro OG, Starobinas N, Cabrera WHK, Ibañez OM, De Franco M
Laboratório de Imunogenética, Instituto Butantan, SP, Brazil

Introduction: Two lines of mice were phenotype-selected for maximal (AIRmax) or minimal (AIRmin) local acute inflammation to polyacrylamide beads (Biogel) injected subcutaneously. Differences between the two lines are found in the exudate protein concentration, neutrophil infiltration and neutrophil resistance to apoptosis, bone marrow granulopoiesis and inflammatory cytokine production during acute inflammatory response. As a result of these alterations, AIRmax mice are more resistant than AIRmin to several bacterial infections and to skin and lung chemical tumorigenesis. In chronic inflammatory diseases such as pristine-induced arthritis, AIRmax are significantly more susceptible than AIRmin mice. **Objective:** The aim of the study was to compare global mRNA expression in bone marrow cells from AIRmax and AIRmin mice without stimulus and during chronic and acute inflammatory reactions. **Methods:** Acute inflammation was induced by subcutaneous injection of Biogel (67%) and chronic inflammation by repeated epicutaneous applications of 1 ug TPA twice a week for 30 days. Microarray analysis was performed using Codelink-GE chips (36k genes) with RNA pools from bone marrow cells of AIRmax and AIRmin control mice and at 24h after Biogel or TPA treatments. The most differently expressed genes were confirmed by quantitative real-time polymerase chain reaction (qPCR) analysis. The over-represented biological themes were investigated using the EASE software. **Results:** Differently (>4-fold) expressed genes were found in normal bone marrow of the two lines. During acute inflammation, there was up-regulation of about 150 and 200 genes in the bone marrow of AIRmax and AIRmin mice, respectively, but about 730 genes were found to be down-regulated in AIRmin compared to 80 in AIRmax. The over-represented biological themes differently expressed between Biogel treated AIRmax and AIRmin mice are biosynthesis and innate immune response with several interleukins, chemokines and their receptors such as IL-1b Il6, Il8rb and Il10, which were confirmed by qPCR. During TPA-induced chronic inflammation, cytokine biosynthesis, synaptic transmission and immune cell activation were the over-represented themes. Moreover, several gene clusters were observed in chromosomes 3, 6 and 11 which co-localized with the regions previously mapped by whole genome screening. **Discussion:** The study of global gene expression in mouse models showing different genetic predisposition to experimental diseases is important to the candidacy of the relevant modifier genes.

Supported by FAPESP, CAPES, CNPq

3.03 mRNA expression analysis in the arthritic joints of mice genetically selected for high (AIRmax) and low (AIRmin) acute inflammation

Peters LC, Borrego A, Carneiro PS, Canhamero T, Garcia LV, Cabrera WHK, Ribeiro OG, Starobinas N, Ibañez OM, De Franco M
Laboratório de Imunogenética, Instituto Butantan, SP, Brazil

Introduction: AIRmax and AIRmin lines homozygous for the *Solute carrier family 11a member 1 (Slc11a1*) *R* and *S* alleles were obtained through selective genotype crosses and were evaluated by the incidence and severity of pristane-induced arthritis (PIA). The AIRmax^{RR} mice reached 29% of PIA incidence, whereas AIRmax^{SS} mice were 70% by day 180. The AIRmin^{RR} mice were completely resistant, whereas 13.3% of AIRmin^{SS} mice became arthritic. The presence of the defective *S* allele also increased arthritis severity. These results indicated that *Slc11a1* or some other closely linked gene interacts with inflammatory QTL to modulate PIA. **Objective:** The aim of the study was to analyze inflammatory gene mRNA expressions in the arthritic joint from AIR mice homozygous for *Slc11a1 R and S* alleles. **Methods:** The AIRmax^{RR}, AIRmax^{SS}, AIRmin^{RR} and AIRmin^{SS} mice received two i.p. injections of 0.5 ml pristane at 60-day intervals, arthritis was assessed for 180 days, and the whole paws were removed. The mRNA was isolated and the expression analysis was carried out with specific primers to the targets and housekeeping genes using SYBR Green Real Time PCR (qPCR). **Results:** The joint mRNA levels of *Tnfα*, *Il1β* and *Il6* were elevated in both AIRmax^{RR} and AIRmax^{SS} mice and showed a significant difference when compared to all the other groups. Interestingly, *Il10* was the only cytokine statistically distinct between AIRmax^{RR} and AIRmax^{SS} mice, showing higher level in the AIRmax^{SS} line. The mRNA levels of *caspase 8* were higher in AIRmax^{RR} and AIRmax^{SS} controls than in the other groups. AIRmax^{RR} mice displayed high levels of *Il8rb* in both control and experimental groups, however differed from AIRmax^{SS} mice. **Discussion:** The arthritic joints of AIRmax expressed high levels of *Tnfα*, *Il1β* and *Il6* independent of *Slc11a1* alleles; such pro-inflammatory cytokines are intrinsically involved in the pathology of rheumatoid arthritis (RA). Higher levels of IL10 related to RA development in humans have been recently reported, corroborating our results concerning *Il10* mRNA levels in the joints of the AIRmax^{SS}. Despite the absence of *Il8rb* gene polymorphisms among the lines, this gene was up-regulated in arthritic joints of AIRmax^{RR}. *Il8rb* is expressed in synovial-tissue macrophages which are involved in angiogenesis and required for the cell recruitment in RA. C*aspase 8* co-localized with *Il8rb* and *Slc11a1* genes, being another candidate gene in this region. In conclusion, the joint expression of *Tnfα*, *Il1β, Il6* and *Caspase 8* in pristane-induced arthritis model seems to be modulated by the acute inflammatory QTL fixed during the selective process, whereas *Il10* and *Il8rb* expressions could be modulated by the interaction of *Slc11a1* alleles with the AIR QTL.

Supported by FAPESP, CNPq, CAPES

3.04 Inflammatory gene expressions in LPS-treated mice selected for acute inflammation

Borrego A, Peters LC, Canhamero T, Garcia LV, Cabrera WHK, Ribeiro OG, Starobinas N, Ibañez OM, De Franco M
Laboratório de Imunogenética, Instituto Butantan, SP, Brazil

Introduction: Lines of mice genetically selected for maximal (AIRmax) or minimal (AIRmin) acute inflammatory reaction differ significantly in susceptibility to infection with *Salmonella* enterica serotype Typhimurium (*S.* Typhimurium) and to LPS sensitivity. Different frequencies of *Slc11a1* alleles, which confer resistance (*RR*) or susceptibility (*SS*) to *S.* Typhimurium, were found in AIRmax and AIRmin mouse lines. To study the *Slc11a1* gene interaction with acute inflammatory QTL, AIRmax^{RR}, AIRmax^{SS}, AIRmin^{RR} and AIRmin^{SS} sublines were produced. AIRmax^{RR} were found to be extremely sensitive to LPS, contrasting with their higher resistance to *S.* Typhimurium infection, while the AIRmin^{SS} mice were the more resistant to LPS effects. **Objectives:** The aim of the study was to investigate the inflammatory gene mRNA expressions and the cytokine serum productions in LPS-treated AIR mice. **Methods:** Two groups of AIRmax^{RR}, AIRmax^{SS}, AIRmin^{RR} and AIRmin^{SS} mice were injected i.v. with 20μg or 80μg of LPS. One group was sacrificed 40 min after challenge and bone marrow cells recovered from femurs. Total RNA was isolated from stimulated and non-stimulated cells. Target mRNA (1μg) was reverse transcribed. Real time PCR (qPCR) reactions were performed with Platinum SYBR Green and specific primers. Target mRNAs were normalized with the expression of cyclophilin house keeping gene and the x-fold up/down-regulation was calculated by comparing stimulated with non-stimulated cells ($2^{-\Delta\Delta Ct}$). In the other group, blood was collected 90 min after stimulus and serum cytokine levels were determined by ELISA. **Results:** LPS(80μg) -treated AIRmax^{RR} mice showed in the serum more IL6 (200 ng/ml) than did AIRmax^{SS} (65 ng/ml), and TNFα production was higher in the AIRmax^{SS} (230 ng/ml) when compared with AIRmin^{SS} mice (85 ng/ml). No differences among the lines were observed for IL10 and IL12p40 productions. The control mice showed undetectable serum cytokine levels. Inflammatory gene mRNA expression in bone marrow cells from animals stimulated with LPS was studied. LPS (20μg)-treated AIRmax^{RR} mice showed 2.0-fold more *Tnfα* gene expression when compared with AIRmax^{SS} and AIRmin^{RR}. AIRmax^{RR} showed 5.0-fold more *IL10* gene expression than did AIRmax^{SS}. *Cd14* and *Lbp* genes involved in septic shock were also analyzed. LPS (80μg)-treated AIRmax^{RR} showed 3.0-fold more *Lbp* expression than did AIRmin^{RR} and 2.0-fold more compared to AIRmin^{SS} mice. The *Cd14* expression was 1.5-fold more in AIRmax^{SS} and AIRmin^{RR} when compared to AIRmin^{SS} mice. **Discussion**: The interline differences in serum IL6 and TNFα production suggest the susceptibility to LPS endotoxic shock is modulated by the interaction of *Slc11a1* alleles with the inflammatory QTL. The elevated *Tnfα* gene expression in AIRmax^{RR} is associated with the higher LPS sensitivity of this line. The significant differences observed for *Cd14*, *Lbp* and *IL10* expression indicated that these genes could be involved in the LPS differential sensitivity observed among the lines.

Supported by FAPESP, CNPq

3.05 Differential susceptibility to polycyclic aromatic hydrocarbon-induced hematotoxicity in mice genetically selected for high or low acute inflammatory response

Katz ISS, Albuquerque LL, Siqueira DM, Souza VRC, Cabrera WHK, De Franco M, Ibañez OM, Ribeiro OG
Laboratório de Imunogenética, Instituto Butantan, SP, Brazil

Introduction: Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants that induce hematological alterations. In mice, *in vivo* PAH treatment decreases bone marrow (BM) and spleen cellularity resulting in an immunosuppressive state. This process involves the metabolism of the PAHs that depends on the activation of the aryl hydrocarbon receptor (Ahr). Differential susceptibility to PAH was described in inbred lines of mice. C57BL/6 susceptible mice showing a high affinity Ah receptor possess the allele called Ahr^{b1}, while the resistant DBA2 mice that show a low affinity receptor possess the allele called Ahr^{d}. Two lines of mice genetically selected for maximal (AIRmax) or minimal (AIRmin) local acute inflammatory response (AIR) to a non immunogenic substance (polyacrylamide gel) differ in susceptibility to carcinogenesis induced by PAHs, such as DMBA. **Objectives:** In this study we examined the effects of DMBA and benzene metabolites on BM and circulating cell numbers in AIRmax and AIRmin mice, the allelic polymorphism of the *AHR* gene, and the mRNA expression of Ahr and *CYP1* family members in the bone marrow, following activation by PAHs in these selected mice. **Methods:** AIRmax and AIRmin mice were treated with a single ip dose of 50mg/kg DMBA in olive oil or 75mg/kg phenol and hydroquinone in PBS for 3 days twice a day. The BM and blood cells were identified and enumerated. Proliferation index of BM and spleen cells was determined in response to GM-CSF + all-trans-retinoic acid and to LPS or ConA, respectively after *in vivo* treatment with DMBA. Total RNA was extracted from bone marrow cells after DMBA treatment to quantify the expression levels of the *AHR, CYP1B1* and *CYP1A1* genes by real-time PCR. **Results:** AIRmax and AIRmin mice showed a complete segregation of the low affinity *AHR* allele (AHR^{d}) and of the high affinity *AHR* allele (AHR^{b1}), respectively. We observed a BM hypocellularity produced by DMBA in AIRmin mice at 12 and 24 hours post treatment, reflecting on the circulating leukocyte number. The treatment with PN/HQ induced a significant BM hypocellularity in both AIRmax and AIRmin mice when compared to control groups treated with PBS. An increase in *CYP1A1* and *AHR* expression in AIRmin at 12h and a suppression in AIRmax BM cells were observed after 24h of DMBA treatment. Myeloid cells from DMBA-treated AIRmin mice showed impaired proliferation after *in vitro* GM-CSF/retinoic acid treatment, whereas cells from AIRmax mice demonstrated normal proliferation. The same profile was observed in spleen cell cultures after LPS stimulus. **Discussion:** The loss of hematopoietic and circulating cells may be due to the selective effects of the PAHs or their metabolites on individual cell types by direct toxicity to the myeloid precursors. AIRmax and AIRmin mice are equally susceptible to the toxic effects of the benzene metabolites (phenol and hydroquinone) which bypass the AHR activation. On the other hand, DMBA induces BM depletion in AHR^{b1} AIRmin mice only. AHR and mostly CYP1A1 mediate the toxicity of DMBA for AIRmin BM cells. Finally, the *AHR* locus may be associated with the genetic control of the inflammatory reaction.

Supported by FAPESP

3.06 Comparative study of macrophage activity in mice genetically selected for acute inflammatory reaction

Arruda AGF, Ribeiro OG, De Franco M, Cabrera WH, Ibanez O, Starobinas N
Laboratório de Imunogenética, Instituto Butantan, SP, Brazil

Introduction: Macrophages are one of the most important cells present in the inflammation environment; they are a source and target of several cytokines, chemokines and other signaling molecules. A good experimental model to study the genetic influence on the inflammatory response are the lines of mice genetically selected for maximal (AIRmax) or minimal (AIRmin) acute inflammatory reactivity. **Objectives:** Macrophage activation of AIR mice were studied as phenotypes of inflammation, using as parameters phagocytic activity and secretion of pro-inflammatory cytokines. **Methods:** Peritoneal macrophages obtained from AIRmax and AIRmin mice previously treated with i.p. injection of thioglycolate or PBS were activated or not with LPS, and then the phagocytosis phenotype was assessed using zymosan particles, or viable or dead polymorphic cells. We also quantified the expression of pro-inflammatory cytokines by ELISA and real time RT-PCR. **Results:** Regarding phagocytosis of zymosan particles, the results showed that macrophages from both lines had the same capacity. When necrotic, apoptotic or viable polymorphic cells were used as particles, the phagocytosis of altered cells was greater than viable cells and the incubation of LPS induced an even higher phagocytic activity of these dead cells. Preliminary analysis of pro-inflammatory cytokine expression revealed that cells from AIRmax mice secreted more IL-1β and TNFα after stimulation with zymosan *in vitro*; however, cells of AIRmin mice produced higher levels of IL-6. When activated with LPS, macrophages of AIRmin mice secreted higher levels of IL-10 when compared with controls and cells from AIRmax mice. **Discussion:** The results showed that AIRmax and AIRmin macrophages have their phagocytic capacity improved after the stimulus *in vivo,* although no differences between the lines were found. However, regarding cytokines, different profiles of secretion were observed in cells of these lines, suggesting distinct mechanisms of activation.

Supported by FAPESP

3.07 HPV16, HPV18 and HPV33: construction of an expression vector for L1 gene cloning

Campo JF[1,2], Coimbra EC[1,2], D'arc M[1,2], Souza HM[1,2], Carvalho JC[1,2], Stocco dos Santos RC[3], Beçak W[1,3], Freitas AC[1,2,3]

[1]Departamento de Genética, Universidade Federal de Pernambuco, PE, Brazil; [2]Laboratório de Genética, Instituto Butantan, SP, Brazil; [3]Setor de Biologia Molecular, LIKA, Universidade Federal de Pernambuco, PE, Brazil

Introduction: Papillomaviruses are causal agents of cancer in mammals, including humans. The development of efficient vaccines is a challenge for the prevention and control of the virus infection and related tumors. In the prophylactic vaccines, the virus capsid proteins, L1 and L2 provide epitopes for neutralizing antibodies. These antibodies can also be induced by similar virus structures, lacking the virus genome, *Virus-like particles* (VLP). These structures can be spontaneously constructed following L1 expression in heterologous systems, such as *Pichia pastoris.* **Objectives:** The aim of this study was to obtain specific constructions searching HPV L1 expression concerning the different virus types: 16, 18 and 33. **Methods:** L1 gene sequence was amplified using the HPV 16, 18 and 33 genomes as templates, cloned into pGEM (Promega), and subcloned into pPICZA (Invitrogen). The correct insert direction was verified by PCR, digestion and sequencing, and the procedures for transforming the host cells were performed. **Results and Discussion:** The insert direction was verified as being correct in all the constructions, and the expression of the target protein is being evaluated with regard to the efficacy of the transformation procedure.

Supported by CNPq, FAPESP, FACEPE, Fundação Butantan

3.08 BPV4 L1 gene expression in *Pichia pastoris*

Souza HM[1,2], Campos JF[1,2], Coimbra EC[1,2], Jesus ALS[1,2], Eugênio RAB[1,2], Cordeiro MN[1,2], Torres FAG[3], Stocco dos Santos RC[4], Beçak W[1,4], Freitas AC[1,2,4]
[1]Departamento de Genética, [2]Setor de Biologia Molecular, LIKA, Universidade Federal de Pernambuco, PE, Brazil; [3]Laboratório de Biologia Molecular, Universidade de Brasília, DF, Brazil; [4]Laboratório de Genética, Instituto Butantan, SP, Brazil

Introduction: Papillomavirus (PV) are small DNA viruses that infect mucosal and cutaneous epithelium and cause benign lesions in human and animals. Occasionally, these lesions can progress to cancer under appropriate environmental conditions. The human papillomavirus (HPV) is associated with many different types of cancer with approximately 450,000 newly diagnosed cases each year and a 50% mortality rate. The bovine papillomavirus (BPV) is associated with papillomatosis and cancer. Cattle cutaneous papillomatosis is not only a health problem but also has economic consequences. Occasionally, entire herds have to be culled if the papillomatosis does not regress. Infections with papillomatosis induce type-specific immune responses, mainly directed against the major capsid protein, L1. Based on the propensity of the L1 protein to self-assemble into virus-like particles (VLPs), our group has conducted some research concerning the development of BPV and HPV vaccine strategies. **Objectives:** This work focuses on the expression of L1 HPV genes in the *Pichia pastoris* expression system for VLP production. **Methods:** L1 gene sequence was amplified using BPV4 genome as template, preliminarily cloned into pGEM-T Easy (Promega), and subcloned into pPICZA (Invitrogen). *P. pastoris* cells were transformed using pPICZAL1B4. The clones obtained were selected for expression, exposed to methanol, and evaluated with dot blot using anti-His antibody. **Results and Discussion:** 300 clones were obtained, 94 were selected for expression and 47 were positive for the production of the target protein (BPV4L1) in the expression system. The selected clones are being evaluated for suitability in VLP production.

Supported by CNPq, FAPESP, FACEPE, Fundação Butantan

3.09 BPV 1 E5 gene cloning and maintenance of the sequences: first steps for expression in *E. coli*

Sircili MP[2], Maranduba CM[1], Carvalho RF[1,3], Giovanni DNS[1], Miranda PM[1], Mendonça RZ[4], Beçak W[1,5], Stocco dos Santos RC[1], Freitas AC[1,5]

[1]Laboratório de Genética, [2]Laboratório de Bacteriologia, [4]Laboratório de Imunologia Viral, Instituto Butantan, SP, Brazil; [3]Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brazil; [5]Departamento de Genética, Universidade Federal de Pernambuco, PE, Brazil

Introduction: Papillomaviruses are associated with different carcinogenesis processes in human and other animals. In cattle, bovine papillomavirus (BPV) is the etiologic agent of neoplasms of the upper gastrointestinal tract and urinary bladder. Some types of papillomavirus can also infect fibroblasts and induce fibro-epithelial tumors, including BPV types 1 and 2, which cause benign fibropapillomas in cattle. Both viruses have a genome of 7900 bp of double-stranded DNA, with at least nine potential reading frames. The early (E) region, encodes the transforming proteins E5, E6 and E7, and the replication and transcription regulatory proteins E1 and E2. In papilloma formation, for example, the virus infects initially the basal keratinocytes. The early region genes are then expressed in the undifferentiated basal and suprabasal layers. Initiation of malignant transformation is linked to the deregulated expression of the early virus genes, which results in an uncontrolled proliferation (and loss of differentiation) of the infected cells. E5 and E6 are the transforming proteins of BPV. The major BPV-transforming protein, E5, is a short hydrophobic membrane protein localizing to the Golgi apparatus and other intracellular membranes. Since the E5 protein is likely to play a role during the early tumorigenesis stage, a vaccine to target E5 may be a good strategy. **Objectives:** In order to improve the development of a vaccine targeting E5, our aim was to clone the BPV1 E5 gene and to verify the maintenance of the sequences. **Methods:** The BPV-1 E5 was amplified by PCR using an upstream primer that included an XhoI restriction site and the most downstream primer a KpnI site. E5 codon sequence of BPV-1 was cloned into pGEM-T (Promega), excised with XhoI and KpnI enzymes, and subcloned into pET (Novagen). **Results and Discussion**: E5 codon sequences of BPV-1 were cloned, sequences were aligned using ClustalX 1.83, revealing an identity of 91%. Bitscore and expected value were calculated based on the size of the nr database. The strategy was shown as appropriate for the construction of the vectors and the maintenance of sequence indicated that the procedures are suitable for the efficiency of E5 gene manipulation.

Supported by CNPq, FAPESP, FACEPE, Fundação Butantan

3.10 BPV2 prevalence in cutaneous papillomatosis?

Monteiro VLC[1], Carvalho CCR[2], Coelho MCOC[1], Beçak W[2,3], Stocco dos Santos RC[3], Rieger TT[2], Freitas AC[1,2,4]

[1]Faculdade de Medicina Veterinária, [2]Departamento de Genética, [4]Setor de Biologia Molecular, LIKA, Universidade Federal de Pernambuco, PE, Brazil; [3]Laboratório de Genética, Instituto Butantan, SP, Brazil

Introduction: Papillomaviruses are associated with different carcinogenesis processes in human and other animals. In cattle, bovine papillomavirus (BPV) is the etiologic agent of neoplasms of the upper gastrointestinal tract and urinary bladder. Eight types of BPV have been identified. They are related to cutaneous fibropapillomas and squamous papillomas of the skin. Infection by papillomavirus requires epidermal or mucosal epithelial cells. Initial infection occurs in basal cells and the access to these cells occurs through micro-abrasions caused by various forms of physical trauma. In studies performed in bovines, our group showed that despite the description of papillomavirus as epitheliotropic, its genetic material was found in several different types of tissues (reproductive tract, embryonic tissues, gametes and blood) in a same animal and also in the peripheral blood of its offspring. The association of papillomavirus and bracken fern induces neoplastic processes such as enzootic hematuria. **Objectives:** We investigated the unusually high occurrence of papillomatosis in a dairy herd in Pernambuco, where the presence of bracken fern was not detected. **Methods:** Lesion fragment samples were collected from affected bovines and were investigated for the presence of papillomavirus genome sequences by PCR techniques, using specific primers. The animals were followed up for one year. **Results and Discussion:** We could detect BPV2 genome sequences in the great majority of the samples examined, suggesting that the virus per se cannot act as an etiologic agent for enzootic hematuria, in the absence of co-factors.

Supported by CNPq, FAPESP, FACEPE, Fundação Butantan

3.11. Bovine papillomatosis in dairy herd: preliminary evaluation in Pernambuco, Brazil

Diniz N[1], Melo TC[1], Campos SRC[3], Freitas AC[1], Lima Filho JL[2], Beçak W[1,3], Stocco dos Santos RC[3], Rieger TT[1], Santos JF[1]

[1]Departamento de Genética, [2]Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, PE, Brazil; [3]Laboratório de Genética, Instituto Butantan, SP, Brazil

Introduction: Papillomaviruses are associated with oncogenic processes in mammals. In bovines, although cutaneous papillomatosis could be the first manifestation of the virus, it has been shown that bovine papillomavirus (BPV) can be experimentally transmitted to other animals through blood of infected animals. Bovine papillomatosis is frequent in several regions of Brazil, with dramatic impairment to cattle breeding, but without systematic evaluations of its occurrence. **Objectives:** In Pernambuco, Brazil, we could detect the first dairy herds with severe cutaneous lesions linked to BPV, and our aim was to investigate the type of lesions, the presence of specific BPV types, and the presence of virus genome in the lesions and peripheral blood, and to analyze the presence of chromosome aberrations in lymphocytes. **Methods**: Lesion fragments and peripheral blood samples were collected from affected bovines. Morphological alterations in infected tissues were analyzed through anatomopathologic studies performed in wart fragments. All the samples were investigated for the presence of papillomavirus genome sequences by PCR techniques, and confirmed as being infected by papillomavirus. Blood samples were incubated in RPMI 1640 medium, supplemented with 20% fetal serum and 2% phytohemagglutinin, and kept for 72 hours at 37°C. Colchicine (16ug/ml) was added for 1 h; the material was centrifuged, and hypotonization (KCl 0.075 M) and fixation (methanol: acetic acid 3:1) were performed. The slides were stained with 3% Giemsa in phosphate buffer, pH 6.8, and analyzed with a light microscope. **Results and Discussion:** The anatomopathologic studies showed different severities of lesions, harboring different virus types. The presence of chromosome markers was detected in the cytogenetic evaluations. No co-factor could be identified (such as *Pteridium aquilinum* in the pastures). The results provide further evidence of the endemic situation of BPV infection and the importance of effective control.

Supported by CNPq, FAPESP, FACEPE, Fundação Butantan

3.12 Characterization of female population exposed to HPV and co-factors in a rural region

Timóteo MA[1], Pitol BCV[1], Moran MS[1], Miranda PM[1,2], Encarnação TBC[2], Félix PM[2], Beçak W[2,3], Stocco dos Santos RC[2], Lima AA[1,2]

[1]Escola de Farmácia, Universidade Federal de Ouro Preto, MG, Brazil; [2]Laboratório de Genética, Instituto Butantan, SP, Brazil; [3]Departamento de Genética, Universidade Federal de Pernambuco, PE, Brazil

Introduction: Human papillomaviruses (HPVs) are circular double-stranded DNA viruses of about 7.9 kilobases, reported to demonstrate approximately 100 different genotypes subclassified into high risk (HPV-16, HPV-18, HPV-31) and low risk (HPV-6, HPV-11) categories on the basis of their association with malignant progression. Earlier studies have demonstrated the presence of HPV genome sequences in several human cancers, particularly squamous cell tumors of the uterine cervix, skin, conjunctiva and upper respiratory tract. It has been discussed that compounds found in bracken fern (*Pteridium aquilinum*) can act as co-factors with HPV in cancer development. **Objectives:** The aim of this study was to evaluate some characteristics of the female population exposed to the consumption of *Pteridium aquilinum* and to HPV infection in Ouro Preto, Minas Gerais, Brazil, considering that the exposure to both agents can increase dramatically the risk of cancer. **Methods**: Two strategies were developed: 1- the identification of all Public Health Centers with records of female patients, 2- the development of an anamnesis interview involving a sample of patients in reproductive age, in order to establish the main characteristics of the group: age, residence, smoking, drinking, food, educational level, use of contraceptives, economic levels, professional activities, and medical data. **Results and Discussion:** The data obtained provided a specific profile for the target population: a rural group, middle age, religious, low educational and economic levels and chronic bracken consumer. All these data suggest that a sexual transmitted disease, such as HPV infection, may have a different prevalence when compared with distinct urban groups, where different approaches for counseling and prevention must be used.

Supported by CNPq, FAPESP, Fundação Butantan

3.13 FAP59/FAP64: primers for investigating the presence of papillomavirus in several host species

Giovanni DNS[1], Campos SRC[1], Marins RSQS[2], Pereira SRFG[2], Nogueira DM[3], Beçak W[1,4], Stocco dos Santos RC[1]

[1]Laboratório de Genética, Instituto Butantan, SP, Brazil; [2]Centro de Ciências e Tecnologias Agropecuárias, UENF, RJ, Brazil; [3]Laboratório de Citogenética Animal, UFF, Brazil; [4]Departamento de Genética, Universidade Federal de Pernambuco, PE, Brazil

Introduction: The oligonucleotide primer pair FAP59/FAP64 was designed considering two relatively conserved regions of the L1 gene of a large variety of HPV types so that many viral types could be detected in cutaneous tumors and in normal skin. By means of PCR amplification, FAP59/FAP64 proved to be adequate for detecting the presence of papillomavirus DNA in skin bearing no lesions in several animal species, showing that the virus has an ample distribution in the animal kingdom. **Objective:** The aim of this study was to carry out a preliminary evaluation of the possible presence of papillomavirus in several wild and domesticated animals displaying or not classical signs of the viral infection. **Methods:** DNA extraction was carried out in 16 animal tissue samples, using the QIAamp DNA blood mini kit (Qiagen), following the manufacturer's instructions. These DNA templates were used for PCR amplification, using appropriate previously established negative and positive controls. Samples were obtained from 4 dogs (2 with clinical lesions suggesting papillomavirus infection and 2 from apparently healthy dogs), 4 bovines clinically presenting with signs of papillomavirus infection, as well as 2 ovines, 2 jaguars, 2 ostriches and 2 macaws without clinical signs of infection. The reactions were carried out on a PTC-100 thermal cycler and performed according to [(1)]. The reaction products were visualized by 2% agarose gel electrophoresis. **Results and Discussion:** The DNA samples obtained from the different analyzed species were verified as showing enough quality for PCR procedure. It was possible to detect amplicons with the expected size in samples from all the species studied. Amplification of sequences in bovines, dogs, ovines, jaguars, ostriches and macaws suggests that FAP59/FAP64 can indicate the presence of papillomavirus, even in different clinical situations where asymptomatic animals may harbor the virus in its latent form. However, it must be determined whether this result was obtained due to the presence of species-specific viral types or due to generic viral types.

Reference: 1. Ogawa *et al.,* Journal of General Virology 85: 2191, 2004.

Supported by FAPESP, CNPq, FAPERJ, Fundação Butantan

3.14 Cytogenetic studies in cells obtained from lesions caused by bovine papillomavirus

Trindade C[1], Ferraz OP[1], Assaf SLMR[1], Góes LGB[1], Encarnação TBC[1], Félix PM[1], Dagli MLZ[2], Birgel Jr EH[3], Beçak W[1,4], Stocco dos Santos RC[1]

[1]Laboratório de Genética, Instituto Butantan, SP, Brazil; [2]Departamento de Patologia, [3]Clínica de Bovinos, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brazil; [4]Departamento de Genética, Universidade Federal de Pernambuco, PE, Brazil

Introduction: Bovine papillomavirus (BPV) is a double-stranded DNA virus that is capable of inducing benign lesions (such as warts) in the epithelium. These lesions can develop into tumors when exposed to certain co-factors. This fact results in grave economic losses. In spite of the widespread availability of literature regarding BPV, there have been few studies on cytogenetic characterization of these lesions and on the distribution of viral DNA in the different cell layers comprising the warts. **Objectives**: The aim of this study was to use this type of lesion to develop a primary cell culture in order to evaluate the presence of viral DNA and carry out a cytogenetic analysis. **Methods:** A cell culture was established from a wart biopsy obtained from a bovine by implanting small fragments of tissue in RPMI medium containing 20% fetal bovine serum. PCR methods were developed to identify the BPV types in the samples at different stages of cell culturing. **Results and Discussion:** The PCR products obtained were identified as DNA sequences of BPV types 1, 2 and 4. Cytogenetic studies showed that cells bore the bovine karyotype, with the presence of marker chromosomes, mostly submetacentric, indicative of centric fusion. These results suggest that this cell line can harbor viral DNA *in vitro* and show aberrations similar to those found in peripheral blood, which corroborates the hypothesis that the virus may be harbored in the blood as well and that these aberrations are induced by the virus.

Supported by CNPq, FAPESP, Fundação Butantan

3.15 Cytogenetic studies in bovine papillomas

Campos SRC[1], Caetano HVA[1,2], Carvalho RF[1,2], Lima AA[1,3], Ferraz OP[1], Marins RSQS[4], Giovanni DNS[1], Encarnação TBC[1], Félix PM[1], Trindade C[1], Beçak W[1,5], Stocco dos Santos RC[1]

[1]Laboratório de Genética, Instituto Butantan, SP, Brazil; [2]Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brazil; [3]Escola de Farmácia, Universidade Federal de Ouro Preto, MG, Brazil; [4]Centro de Ciências e Tecnologias Agropecuárias, Universidade Estadual do Norte Fluminense, RJ, Brazil; [5]Departamento de Genética, Universidade Federal de Pernambuco, PE, Brazil

Introduction: Papillomavirus is a dermotropic oncogenic DNA virus described in a great range of hosts. Evidence suggests that the virus is species- and tissue-specific. However, several reports have shown the initiation of lesions in phylogenetically close hosts, the presence of the virus genome in other tissues, non epithelial (including peripheral blood) and the action of the virus on the host chromatin (in human and bovine peripheral blood lymphocytes and in bovine wart cell cultures). **Objectives**: We evaluated chromosome abnormalities in peripheral lymphocytes and cell cultures obtained from warts (papillomavirus-induced benign lesions). **Methods:** Lesion fragments and peripheral blood samples were collected from affected bovines. All the samples were investigated for the presence of papillomavirus genome sequences by PCR, using generic primers, and confirmed as being infected by papillomavirus. Fragments of the tumors were incubated in RPMI 1640 medium, supplemented with 20% fetal serum and kept at 37°C. Blood samples were incubated in RPMI 1640 medium, supplemented with 20% fetal serum and 2% phytohemagglutinin, and kept for 72 hours at 37° C. In both procedures, colchicine (16ug/ml) was added for 1 h; the material was centrifuged and hypotonization (KCl 0.075 M) and fixation (methanol: acetic acid 3:1) were performed. The slides were stained with 3% Giemsa in phosphate buffer, pH 6.8, and analyzed with a light microscope. **Results and Discussion:** All the samples were positive concerning the presence of papillomavirus. The major cellular abnormality observed was the presence of chromosome markers, submetacentric chromosomes, with different levels of condensation, indicating alteration in cell cycle controls and in the centromeric regions. The results provide further evidence regarding the presence of virus genome sequences in non epithelial tissues and its possible expression, resulting in host chromatin damage and also altered cell cycle control.

Supported by CNPq, FAPESP, FAPERJ, Fundação Butantan

3.16 Evaluation of polymorphism of the p53 gene related to HPV and BPV infection

Felix PM[1], Encarnação TBC[1], Trindade C[1], Miranda PM[1,2], Pitol BCV[2], Moran MS[2], Timóteo MA[2], Lima AA[1,2], Beçak W[1,3], Stocco dos Santos RC[1]

[1]Laboratório de Genética, Instituto Butantan, SP, Brazil; [2]Escola de Farmácia, Universidade Federal de Ouro Preto, MG, Brazil; [3]Departamento de Genética, Universidade Federal de Pernambuco, PE, Brazil

Introduction: The p53 protein is a nuclear phosphoprotein whose concentration depends on the cell cycle phase. Wild-type p53 has a role in negatively regulating cell proliferation, preventing an increase in DNA damage. It is expressed when DNA suffers some sort of damage. The p53 protein then binds to DNA at the site where the damage occurred and interrupts the cell cycle in the G1 phase, activating DNA repair mechanisms, possibly inducing apoptosis in the G2 phase or activating several other genes related to the cell cycle. The inactivation of the physiological function of p53 may occur with its conjugation with viral proteins, by the insertion of oncogenes in the host's genome or by stimulating the transcription of cell oncogenes by the insertion of papillomavirus-derived transcription activating sequences. **Objectives:** The aim of the study was to evaluate p53 polymorphism in human and bovine samples infected or not by papillomavirus, in order to establish a correlation between the p53 alterations and virus presence. **Methods:** We obtained blood, lesion and cervical samples from female patients and bovines infected by papillomavirus. The samples were submitted to DNA extraction using the QIAamp DNA blood mini kit (Qiagen), according to the manufacturer's instructions, and to PCR amplification (using primers specific for PV sequences). PCR reactions using specific primers for the detection of p53 polymorphism were carried out according to the manufacturer's instructions. **Results and Discussion:** The results showed a correlation between polymorphic alterations of the p53 gene and the presence of HPV and BPV, with detected mutations in the gene. The p53 mutations can be used for discussion of specific neoplastic alterations related to papillomavirus infection.

Supported by CNPq, FAPESP, Fundação Butantan

3.17 Chromosome aberrations in lymphocytes of HPV infected women

Ferraz OP[1], Encarnação TBC[1], Rieger TT[2], Beçak W[1,2], Stocco dos Santos RC[2], Lindsey CJ[3]

[1]Departamento de Genética, Universidade Federal de Pernambuco, PE, Brazil; [2]Laboratório de Genética, Instituto Butantan, SP, Brazil; [3]Departamento de Biofísica, Universidade Federal de São Paulo, SP, Brazil

Introduction: The presence of HPV in human peripheral blood has been discussed: we have identified BPV2 DNA sequences in bovine peripheral blood. Lymphocytes of these animals, which were afflicted with chronic enzootic hematuria (a malignant disease of the urinary tract) showed a significant increase in the level of structural chromosome aberrations. Lymphocytes are considered a possible latency site for this type of virus, and the virus genome expression was evaluated for its possible clastogenic action. **Objectives:** The aim of the study was to evaluate the chromosome aberrations in peripheral blood lymphocytes obtained from women presenting with HPV, in different clinical situations, exposed or not to co-factors. **Methods:** We analyzed cultured peripheral blood lymphocytes collected from: **1**- 56 breast cancer affected women who also had a history of CIN III, were followed-up, received chemotherapy and/or radiotherapy, and were considered clinically cancer free for at least 5 years before the present study, **2**- 70 women in reproductive age who were chronic consumers or not of bracken fern, showing different levels of cervical lesions or no symptoms at all, **3**- 25 women presenting with HPV-related CIN I and CIN II, and **4**- 22 healthy women with no reports of gynecological diseases or exposure to co-factors, as control. Blood samples were incubated for short-term lymphocyte cultures, and the slides obtained were analyzed with a light microscope for cytogenetic evaluation of structural chromosome aberrations. PCR with specific primers for HPV, sequencing techniques for detection of virus DNA, and ISHI technique for detection of integrated HPV DNA sequence in host chromosomes were performed. **Results and Discussion:** The presence of HPV genome sequences was detected in samples of all the groups, except in the control. Cytogenetic analyses detected a significant increase in cells with structural chromosome aberrations in the patient groups compared with the control group. The major abnormalities were related to telomere associations. Using the ISHI procedure, it was possible to detect a positive signal in the 3p region, suggesting possible integration of HPV sequence in host chromatin. Our findings are discussed in terms of eventual multiple viral infections, possible viral integration to host genome and hidden latency sites. The data point to a major role of lymphocytes in the virus-host interaction and the virus-host chromatin interaction.

Supported by CNPq, FAPESP, Fundação Butantan

4. Biology of snakes, arachnids and amphibians

4.01 Application of neutron activation analysis for trace element determination in plasma, yolk nd embryos of *Bothrops jararaca*

Janeiro-Cinquini TRF[1], Saiki M[2], Alves ER[2], Winter CE[3], Kubrusly FS[4], Veiga JR[4]

[1]Laboratório Herpetologia, [4]Laboratório Biotecnologia Instituto Butantan, SP, Brazil; [2]Instituto de Pesquisas Energéticas e Nucleares, CNEN, [3]Departamento de Parasitologia, Universidade de São Paulo, SP, Brazil

Introduction: There is a great interest to understand the evolution of viviparity of snakes since published studies in this field are scarce and the results sometimes contradictory. As viviparity may involve the supply of nutrients to the growing embryo, analyses of plasma, yolk and embryo tissues have been carried out in the viviparous snake *Bothrops jararaca*. Analyses of element composition in egg yolk have been performed to understand nutrient utilization during embryonic development. **Objective**: The main objective of this study was to quantify the amount of several elements in oocytes and embryos during development, and correlate these variations to variations observed in the plasma of the pregnant female. **Methods:** The snakes were obtained from the Laboratório de Herpetologia of the Instituto Butantan, originating from several locations in São Paulo State, Brazil. For the analyses, the samples were first lyophilized and then aliquots of about 200 mg were weighed in polyethylene vials and irradiated along with elemental standards at the IEA-R1 nuclear research reactor. IEA-R1, Brazil's first research reactor is a light water pool type reactor that has been operating since 1957 at IPEN-CNEN (Instituto de Pesquisas Energéticas e Nucleares; Comissão Nacional de Energia Nuclear) in São Paulo, Brazil. **Results:** We observed during development two peaks of zinc, selenium and rubidium in the plasma, one in the animals that showed only fertilized follicles and another one in those with embryos in more advanced stages of development. The lower levels of these ions in vitellogenic animals indicate an active removal of these ions from plasma by the growing oocytes. The amount of these ions in yolk increase in the vitellogenic phase. In embryos, the amounts of zinc, selenium and rubidium increase irregularly during development, reaching high levels (400 μg/embryo) in neonates. **Discussion:** We show here for the first time, in a neotropical snake, evidence that viviparous viperid placenta is involved in cation exchange between mother and growing young in the uterus. We hypothesize that zinc is transported from the mother to the young by plasma proteins, probably vitellogenin which is known to have two atoms of zinc per molecule.

4.02 Description of the embryonic development stage of *Oxyrhopus guibei* (Serpentes: Colubridae) on the day of its ovoposition

Cavlav CL[1], Almeida-Santos SM[2], Sano-Martins IS[1]
[1]Laboratório de Fisiopatologia, [2]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: *Oxyrhopus guibei*, the common false coral snake, is an abundant oviparous species in forest edges and open areas in Southeastern Brazil. Oviparity mode of reproduction occurs when the female lays fertilized eggs with relatively undeveloped embryos. However, many species of oviparous squamates retain their eggs *in utero* and embryonic development occurs before oviposition. **Objective:** Considering the limited information on the extent of embryonic development in the uterus in neotropical colubrids, the objective of this study was to describe the stage of embryonic development of an egg of *Oxyrhopus guibei*, on the day of its oviposition. **Methods:** With a dissecting microscope, morphological characteristics of the embryo were observed, and these were compared with previously published staging tables for other snakes: Hubert and Dufaure's (L. Bull. Soc. Zool. France, 1968 93: 135-148) staging table for *Vipera aspis*, and Zehr's (Copeia 1962: 322-329) staging table for *Thamnophis sirtalis*. **Results:** The embryo was inside of the closed amniotic cavity; the chorioallantois covered the embryo completely; the cerebral hemispheres were expanded in extension; the optic vesicle was circulated and the eyes were unpigmented; the nasal pit was visible and it was well delimited by the nasal bud, divided by an internasal depression; the mandibular bud was extended below of the eye; the neural tube was opened in the anterior extremity and its was visible due to the transparency in the cephalic region; and the embryo was three-four times coiled inside of the amniotic cavity. Based on these results, it is possible to establish that this embryo of *O. guibei* on the day of oviposition corresponds to 32nd stage of embryonic developmental described by Hubert and Dufaure, and to 23rd stage described by Zehr. **Discussion:** Our results show that *Oxyrhopus guibei* retain its egg in the uterus, where part of the embryonic development occurs. The short period that the embryo of *O. guibei* has environmental exposure may perhaps increase the survival of the offspring in this species.

Supported by CNPq

4.03 Where are the eggs? Nest-sites and oviposition modes of the 'goo-eater' snake *Sibynomorphus mikanii* (Colubridae, Dipsadinae)

Braz HBP, Franco FL, Almeida-Santos SM
Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: For oviparous reptiles, female nest site selection plays a significant role in the evolution of life histories. However, nest sites and modes of egg laying of Brazilian snakes have long been a neglected issue in reproductive ecology. For the most part because snakes are so successful at hiding their eggs, nests are rarely found in nature. Moreover, much emphasis has been placed on life history components as body size, number and size of offspring, and age at maturity. **Objective:** Herein, we describe three nest sites, nesting areas and modes of egglaying of the goo-eater snake *Sibynomorphus mikanii* in southeastern Brazil. **Description of the nests:** Nest #1 (n = 41 eggs) was found within a hole at the edge of Instituto Butantan woods, 2 meters away from a house backyard. Empty shells (n = 11) were also found within the nest. Nest #2 (n = 12 eggs) and #3 (n = 9 eggs) were discovered at a farmyard, beneath a rock pile and beneath a dry grass pile, respectively. Surrounding area of all nests were open areas nearby forests. Eggs were collected and incubated in the laboratory. Based on mean clutch size of this species, nest #1 and #2 were considered communal nests, whereas nest #3 was likely a single nest. Hatching dates supported these suggestions. **Discussion:** The selection by multiple females for the same nesting site in highly disturbed areas may indicate that suitable nesting sites (e.g., optimum soil composition, moisture, and temperature; easy entrance and egress) were scarce. Thermal conditions are often suggested as a factor driving maternal choice of nest site because incubation temperature affects offspring phenotypes. Oviposition in open areas increases the level of insulation of soil and consequently increases temperature within the nest, allowing embryonic development to occur in optimal conditions. Additionally, temperature in communal nests may be higher than in single nests due to metabolic heat generated by embryos during incubation period. Egg aggregations also offer potential advantages such as protection and predator satiation. The presence of old eggshells only in nest #1 may indicate that this site was used in successive years for oviposition. Our findings plus literature data suggest that seemingly, in *S. mikanii,* the presence of humans or dogs nearby nesting areas do not preclude a site from being an appropriate nesting site, even if this site is located at a disturbed area. Nevertheless, the adaptive significance of communal oviposition remains unknown.

4.04 Is hemiclitoris a functional organ? Studies of the size of the hemipenis, hemiclitoris and retractor muscles in *Bothrops insularis*

Kasperoviczus KN, Germano VJ, Almeida-Santos SM
Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: *Bothrops insularis* is a natural snake of Queimada Grande Island and shows in its reproductive biology, anatomic particularities that are observed uniquely in this insular species: female of this species has an identical structure to the male organ (hemipenis). Previous research shows the occurrence of male, female and intersexes. Lately, these intersexes have been recognized as real female and that structure denominated hemiclitoris. Therefore, the term intersex has not been applied in this species. Meanwhile, preliminary analyses indicate the presence of hemiclitoris in all females, which would configure intersexuality, in other words functional organs of other sexes. **Objectives:** The aim of this research was to compare the size of the hemipenis/hemiclitoris and the width of its retractor muscles in *Bothrops insularis*. **Methods:** For that purpose, 73 male and female specimens were dissected. All the females examined possessed a hemiclitoris. In each specimen, we measured the size of the hemipenis/hemiclitoris (mm), from the base of the cloaca until the point of insertion where the retractor muscles starts. We also measured the retractor muscle width (mm). **Results and discussion:** The measurement of the right hemipenis was 17.4 mm ± 4.14 and the left was 16.9 mm ± 3.05 ($t=0.82$, $p> 0.05$, $n= 42$) while for the hemiclitoris on average was 12.1mm ± 2.95 and the left was 10.0 mm ± 1.00 ($t=2.74$, $p<0.001$, $n=31$). The results indicate that the comparison of the size of the hemipenis right/left side of the male is not significant, while the female hemiclitoris on the right/left side is significant. Meanwhile, both the right hemipenis of the male, and the right hemiclitoris of the female, are larger, and this could indicate the dexterous ability and functionality of this side of the organ. Males could utilize during mating mainly the right hemipenis, while the larger size found in the female require more studies. The average width of the right/left side of the retractor muscles of the male was 2.09 mm ± 0.57 and 1.98 mm ± 0.53, respectively ($t=0.60$, $p>0.05$, $n=19$), while for the female the average size found on the right/left side was 1.17 mm ± 0.31 and 1.00 mm ± 0.27, respectively ($t=2.23$, $p< 0.01$, $n=31$). These values are similar to those found in hemipenis/hemiclitoris, so larger organs need larger and stronger muscles, because it depends on it to retract it and to put it back to its normal position after mating, besides the resistance of these to support the organ during the mating that can last for many hours. Therefore, the information indicates that the larger the organ, the thicker the caliber of the retractor muscles associated, indicating or not its functionality. At the same time, it is evident the difference between sizes of hemipenis and hemiclitoris and also between the width of its retractor muscles, that in males they are larger and more developed than the female. Even though being smaller, the hemiclitoris and the retractor muscles follow the same pattern of measurement of the hemipenis. Therefore, more research is necessary to prove the functionality or not of these organs in the female.

Supported by FAPESP

4.05 Reproductive biology and features of annual and daily activity of *Leposternon microcephalum* (Squamata, Amphisbaenidae) from Southeastern Brazil

Santos LC[1,2], Almeida-Santos SM[1]
[1]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil; [2]Instituto de Biociências, Universidade de São Paulo, SP, Brazil

Introduction: The Amphisbaenia, popularly called two headed snakes, are fossorial Squamata. There are few studies about the habits and biology of the group. In part, that can be attributed to their fossorial habits, which make field work difficult, and explains the low number of specimens in zoological collections. *Leposternon microcephalum*, from the family *Amphisbaenidae*, is a species wide spread in Brazil, especially on the area once occupied by the Atlantic Forest. **Objectives**: The aim of this work was to study the reproductive biology of *L. microcephalum* from Southeastern Brazil, describing aspects of the reproductive cycle of males and females, analyzing the maturity length and the existence of sexual dimorphism in the length of the body. We also aimed to analyze patterns of daily and annual activity of the species, and the relations of those patterns with environment variables. **Methods**: We dissected fixed animals to examine the morphological aspects of the testis, vas deferens, follicles and oviducts, and measured those organs. We assumed that turgid testis, opaque and convoluted vas deferens, yellowish follicles and opaque and pleated oviducts indicate the animal to be reproductive. With dissection data, we estimated the seasons of secondary vitellogenesis, sperm production, and mating. Afterwards, we started to study the histological appearance of reproductive organs, to compare it with morphological data, completing our analysis about the reproductive cycle, and to make a histological description of the reproductive trait of the species. We analyzed the existence of sexual dimorphism in snout-vein length (SVL) and tail length utilizing Student's t-test. Also utilizing SVL and dissection data, we evaluated maturity length of males and females. Daily and annual activity patterns were studied using data from animal filming in captivity and from specimen reception at Instituto Butantan. The data were compared with maximum and minimum temperature and precipitation data, utilizing multiple regressions. **Results**: The patterns revealed on reproductive cycle graphics show greater testis size in September and larger and vitellogenic follicles in November and December. There was no significant variation in vas deferens diameters during the year. Histological data, up to now, are confirming morphological data in determining maturity. Convoluted vas deferens, turgid testis, and yellowish follicles seemed to be for sperm storage, sperm production and secondary vitellogenesis, respectively. The pattern of annual activity shows two peaks per year, one of them apparently related with mating season. Daily activity pattern demonstrated that the species is active mainly during the day. Both annual and daily activity was shown to be related to environmental temperature. We did not find sexual dimorphism in the length of the body in this species. **Discussion**: There is an imbalance between the probable seasons of testicular recrudescence and follicular maturity. It is possible that there is little sperm storage in that period, and that mating occurs in December. This sperm storage seems to be indicated by convoluted vas deferens, but not by variations in that organ's diameter. Temperatures lower than 20ºC seem to be non permissive for this species' activity.

Supported by FAPESP

4.06 Reproductive biology of *Liotyphlops beui* (Anomalepididae) in southeastern Brazil

Parpinelli L, Marques OAV
Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: Scolecophidia include three families: Leptotyphlopidae, Tyhlopidae and Anomalepididae. The Anomalepididae occur only in the Neotropics and information on its natural history is absent. In this family, *Liotyphlops beui* is abundant in collections. **Objectives:** The aim of the study was to describe the reproductive cycle of *L. beui* as well as possible in relation to habitat use in this group of snakes. **Methods:** We used specimens preserved in the collection of the Laboratório de Herpetologia of Instituto Butantan, São Paulo, Brazil. Each specimen was dissected with a midventral incision. Measure of ovarian follicles, oviductal eggs, oviductal egg numbers, characteristics of testes and vas deferens were obtained. This data was compared with collection dates to describe the reproductive cycle. **Results:** The smaller mature female is 227 mm and the smaller mature male 219 m in snout-vent length. Females have highly seasonal reproductive cycles, with vitellogenesis (follicles larger than 2.5mm) between August and January. No females with eggs were found. Males have an increase in testis volume between March and May. The vas deferens is wider between September and January. **Discussion:** Like other Scolecophidia**,** females showed secondary vitellogenesis between September and November (rainy season). Absence of eggs in females may be due to the fact that they remain in secretive habitat during this period. Males showed larger testes during the dry season and wider vas deferens during the rainy season. This asynchrony between follicle cycles and testes indicate an uncoupled reproduction with possible sperm storage. In this case, mating can occur in September and October. *Liotyphlops beui* is more active during the rainy season (September – March), with peak in March (pers. obs.). Activity in rainy season can reflect the mate search, parturition and thermoregulatory behavior, whereas peak activity in March may be related to males' thermoregulatory behavior.

Supported by CAPES

4.07 Termite nests as brood-laying sites by *Crotalus durissus*

Travaglia-Cardoso SR, Almeida-Santos SM
Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: There are widely disseminated reports of lizards, snakes and amphisbaenians ovipositing in the nests of termites and ants. These species lay their eggs or broods making use of the constant temperature and humidity found inside them. The temperature of the inner chambers of these structures has been found to be stable at 27-29°C. **Objectives:** We provided the first account of a viviparous snake *Crotalus durissus* using termite nests for brood provision, and this report suggests communal nesting for one female with young and other gravid female in a termitarium. Herein, we describe three nest sites of the *Crotalus durissus* in southeastern Brazil. **Description of the nests:** Termite nest 1: in January, two adult females (one gravid) and ten young were found in Botelhos (46°23'W; 21°38'S), state of Minas Gerais. During the transport to Instituto Butantan eight snakes were born. Termite nest 2: in March, one female was collected inside of a termitarium in Amparo (46°45'W; 22°42'S), state of São Paulo. Also, during transport to Instituto Butantan, the snake gave birth to eleven young. Termite nest 3: in January, one gravid female (1010mm in snout-vent length (SVL); 1025g in mass) was found in the Munhoz, Santa Elisa Farm (22° 36´S; 46°14´W), state of Minas Gerais, inside a termite nest of approximately 1.0 m in diameter. After seven days, the female gave birth to seven live young (four males; three females) and three stillborn. **Discussion:** Termite nests can average 5–11°C higher than outside air temperatures because they are usually oriented in such a way as to catch the rays of the sun in the early morning and late evening, when air temperatures are low. In reproductive strategies, viviparous snakes utilize termite nests for broods because they afford a number of obvious advantages (e.g., constant humidity and temperature) and the increased temperature in termite nests allows embryonic development to occur under optimal conditions. Other advantages of such sites may include a physical barrier and a biological defense against predation. Our preliminary results show that the habit of using termitaria for brood provision seems to be common in *Crotalus durissus*.

4.08 Reproductive aspects of *Bothrops leucurus* (Serpentes, Viperidae) – Male combat, mating and litters

Sant'Anna SS, Nicoleti AF, Betkowski SE, Grego KF, Fernandes W
Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: *Bothrops leucurus* is a pit viper from Bahia State that inhabits broadleaf evergreen forests at altitudes below 700 m. It is also found in the states of Sergipe, Alagoas and Ceará. This species is very common in the Atlantic Forest and is responsible for many of the snakebites occurring in Bahia. Although its venom is not used to produce the botropic antiserum, some specimens are maintained at the Laboratory of Herpetology at Butantan Institute (LHIB) for reproduction means and biomedical research. *B. leucurus* together with *B. asper*, *B. atrox* and *B. moojeni*, compose the atrox group. Each species of this group, although phylogenetically related, occupies different habitats. **Objective:** This study is part of a larger project on reproductive husbandry of Brazilian venomous snakes maintained in captivity, aimed at making the LHIB self-sufficient. **Methods:** Nine males and eight females from Southern Bahia, kept in captivity at the LHIB, were used in this reproductive study. The experiment was carried out by releasing two males on the room's floor or inside a glass terrarium thirty minutes before releasing the female. The reproductive behavior of the snakes was written on standard forms and films and photographs were taken. After 24 hours the animals were returned to their plastic boxes, and the females observed until the birth of the offspring. **Results:** Eleven breeding attempts were made between the months of May and August from 1999 to 2005. In eleven breeding attempts, nine were successful. Male combat was seen in five breeding attempts, and the larger males were always the winners. Not all copulations were preceded by male combat, and in these cases the smaller males had the chance to mate. Offspring were born from December of the same year until February of the next one. A total of 81 males and 93 females were born from seven analyzed clutches (mean number of offspring/clutch = 25, range 7-36). There was no significant difference in the snout-vent length (SVL) and weight of the different sexes. The mean SVL and weight of offspring were 23.9 cm and 8.0 g, respectively. The smallest female which gave birth measured 113 cm and the mean relative clutch mass (RCM) was 23%. **Discussion**: The maintenance of *B. leucurus* in captivity at LHIB has been efficient as we had success in 80% of the matings and births. The ratio of offspring sex was almost 50% and there were no difference in the SVL and weight of the male and female offspring, similar to the pattern encountered in other species of the same genus. Like other viperids of the *atrox* group, *B. leucurus* also displays male combat before mating, although this behavior is not essential for reproduction.

4.09 Reproduction of *Bothrops atrox* (Serpentes, Viperidae) in captivity

Sant'Anna SS, Fernandes W, Barros MC, Bauer A, Grego KF
Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: *Bothrops atrox* is a more common pit viper from the north part of South America. This species has a wide distribution, occurring in Venezuela, throughout the Guianas, in Amazonian Brazil, Colombia, Peru, Ecuador and Bolivia. *B. atrox* occupies a variety of habitats, preferentially damp environment like rainforest, tropical deciduous and gallery forests, and also anthropical areas. This specie is responsible for 80% of the snake accidents in Amazonas state. Although its venom is not used to produce the botropic antiserum, some specimens are maintained at the Laboratory of Herpetology at Butantan Institute (LHIB) for reproduction means and biomedical research. **Objective:** This study is part of a larger project on reproductive husbandry of Brazilian venomous snakes maintained in captivity, aimed at making LHIB self-sufficient. **Methods:** Ten males and six females from the states of Amazonas and Maranhão, kept in captivity at the LHIB, were used in this reproductive study. The experiment was conducted by releasing two males on the room's floor or inside a glass terrarium thirty minutes before releasing the female. The reproductive behavior of the snakes was written on standard forms and films and photographs were taken. After 24 h, the animals were returned to their plastic boxes and the females observed until the birth of the offspring. **Results:** Twelve breeding attempts were made between the months of April and September from 1997 to 2004. In 12 breeding attempts, five were successful, but only four clutches were born. Male combat was seen in six breeding attempts and the larger males were the winners four times. Only one copulation was not preceded by male combat and in this case a much smaller male had the chance to mate. The male combat lasted from five min until 2 h and 3h 50 min, but in these latter cases with long intervals. Offspring were born from November of the same year until April of the next one. Two clutches had only two live snakes, but 14 and 34 eggs. One litter had 11 and another 27 live young (23 females and 15 males). There was no significant difference in the snout-vent length (SVL) and weight of the different sexes. The mean SVL and weight of offspring were 23.9 cm (range 23.0-24.5 cm) and 9.6 g (range 8.5-10.1g), respectively. **Discussion**: Like other viperids of the *atrox* group, *B. atrox* displays male combat before mating and the seasons of mating and recruitment in captivity maintain the same pattern as in nature. The birth of two clutches with only two offspring and the large number of atresic eggs expelled shows the necessity to improve the reproduction system of *B. atrox* in captivity. More studies about reproduction of this species are necessary, such as in modifying the mating period, improving the physiological conditions of females and males and changing other variables to increase the recruitment of *B. atrox* in our animal facility.

4.10 Data on litters of pit viper *Bothrops moojeni* (Serpentes, Viperidae)

Fernandes W, Grego KF, Pisciolaro AM, Sant'Anna SS
Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: Knowledge of the reproductive biology of the Neotropical pit vipers is incipient. A larger amount of data about the number of animals per clutch, the relation between the size of the litter and the mother's size, as well as other reproductive aspects are needed so that intra- and inter-specific comparisons can be made. In Brazil, *Bothrops moojeni* occurs in central and southeastern parts of the Cerrado, such as gallery forests and adjacent wet grasslands. Its venom is used to produce the botropic antiserum, and some specimens are then maintained at the Laboratory of Herpetology at Butantan Institute (LHIB) for extraction, reproduction means and biomedical research. **Objective:** This study is part of a larger project on reproductive husbandry of Brazilian venomous snakes maintained in captivity, aimed at making LHIB self-sufficient. **Methods** Information on reproductive aspects of the *B. moojeni* were obtained from the birth of 15 clutches of recently caught females complemented with two matings and births in captivity. For mating, two males were released in the room and after 30 min a female was released or a group (3 males and two females) was released in a serpentarium (10 x 5 m). The reproductive behavior was observed. **Results**: Only three positive results (three clutches) were obtained with 13 attempts of mating in December 1995 and between the months May and August from 1998 to 2006. Male combat was seen in five breeding attempts and sometimes a male bit the other. The attempts in December were very successful, and the litters were born in November and December of the following year. The average snout-vent length (SVL) and mass of litters were 24.2 cm (n= 248, range 15.5 – 28.5 cm) and 9.5 g (n= 248, range 3.4-16.0 g), respectively. Males (n=102) and females (n=145) were born with the distal portion of the tail of clearer coloration, and the males had a longer tail than the females. The smallest female that gave birth measured 99 cm of SVL and the number of snakes per litter varied 3-34 (mean number of offspring/clutch = 14.3), where there was a positive relation between the size of the mother and the number of offspring. **Discussion:** Although December is not a known reproductive season, the two matings that occurred in this month were successful. This shows that our knowledge on the natural history of this genus is incipient and more studies about reproduction are necessary, such as modifying the mating period, improving the physiological conditions of females and males and changing others variables to increase our knowledge on reproductive plasticity in captivity.

4.11 Comparative morphology and biology of two Brazilian snake species of Elapormophini tribe (Serpentes: Colubridae)

Herédias-Ribas CM, Sawaya RJ
Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: Natural history provides basic information for different areas of biology. Scientific collections permit good characterization of snake natural history and ecology. The tribe Elapomorphini (Colubridae, Xenodontinae) comprises the genera *Apostolepis*, *Elapomorphus* and *Phalotris*. The members of this group display morphological differences. Its phylogeny is relatively well known but data on its natural history is virtually unknown. **Objectives:** We proposed a comparative study of morphological and biological characters of *Elapomorphus quinquelineatus* and *Phalotris mertensi* from preserved specimens deposited in scientific collections. **Methods:** We recorded the following data for each specimen: snout-vent length (SVL); tail length (TL); head length (HL); mass; sex; reproductive maturity; diameter of largest ovarian follicles or oviductal eggs; volume (by length, height, and width) of testes; and diameter of the vas deferens near the cloaca. Sexual dimorphism in SVL was tested by Student's *t* test, and sexual dimorphism in stoutness (relative mass), relative TL, and relative HL were tested by ANCOVA (all variables natural log-transformed), with total length, SVL, and trunk length (SVL - HL) as covariates, respectively. **Results:** We found no significant differences between sexes in relative head length for two species. For both species, males have larger relative tail length and are stouter, but females are larger in snout-vent length. In both species, females attained sexual maturity with larger SVL than males. Interspecific comparisons indicate *P. mertensi* as being larger in SVL and relative tail length than *E. quinquelineatus*. **Discussions:** Snake morphology may be related to natural history traits, such as reproduction and habitat use. Larger bodies in females may be related to larger clutch size and fecundity, with females reaching maturity with enough energy reserve for producing and carrying eggs. Males could be stouter than females because they need energy reserves for spermatogenesis. However differences in stoutness between sexes could be related to differences in habitat use. Sexual dimorphism in the relative head size is common when diet divergences are present. The lack of difference between sexes in relative head size indicates that males and females probably feed upon similar prey items of similar sizes. Males show larger tails because of the presence of a hemipenis and its retractor muscles. Although they are closely related phylogenetically, the two species show differences in morphological traits. Both species are fossorial, but the differences in morphology could be related to subtle differences in habitat use. Acknowledgements: We thank: F. L. Franco, R. Feio, R. Scartozzoni, M. Rodrigues, V. Germano and many other people from Laboratório de Herpetologia for assistance in the laboratory.

Supported by CAPES

4.12 Feeding adaptations related to the infralabial glands in the snake *Dipsas indica*: a behavioral and morphological study

Oliveira L[1], Antoniazzi MM[1], Prudente ALC[2], Zaher H[3], Jared C[1]
[1]Laboratório de Biologia Celular, Instituto Butantan, SP, Brazil; [2]Museu Paraense Emílio Goeldi, PA, Brazil; [3]Museu de Zoologia, Universidade de São Paulo, SP, Brazil

Introduction: Among feeding specializations in snakes, those involved in mollusk ingestion stand out. The species of genus *Dipsas* have a diet based on snails, extracting these mollusks entirely from the shell without breaking it. Although the morphology and physiology of these glands are very poorly known, the large size of the infralabial glands in *Dipsas* has been frequently associated with feeding habits. **Objectives:** The aim of the study was to observe the feeding behavior of *Dipsas indica*, related to the morphology of its infralabial glands. **Methods**: The feeding behavior was recorded in captivity by nocturnal filming using two charge-coupled device (CCD) cameras. The position of the gland in the mandible and its relation to the subjacent musculature was observed by dissections using a stereomicroscope. The dissected glands and entire decalcified heads were prepared for histology and histochemistry. **Results:** The snake captures the snail using a strike to immobilize it while introducing the mandible inside the shell. A series of alternating mandibular movements rapidly extract the mollusk from the shell, which is then released by the snake. The infralabial gland is divided into two portions. The more developed portion is located ventrolaterally to the dentary and composed bones, and is covered by the *levator angulioris* muscle. The smaller and thin portion is hardly observed during dissection, running along the lip, just under the infralabial scales. Mucous cells arranged in tubules mainly comprise the glandular larger portion; acini constituted by seromucous cells are restricted to the peripheral and posterior glandular region. A second type of seromucous cell was identified, associated with both tubules and acini. Departing from the larger portion of the gland, two main ducts transport the secretion to the anterior region of the mouth. **Discussion**: Unlike the infralabial glands of other snakes, which usually show a simple elongated shape and are only associated with mucous secretion, in *Dipsas indica* these glands have two distinct portions, one of them very well developed and composed of cells secreting mucous and protein material. The ducts are directed to the anterior region of the mandible, which is inserted inside the shell during mollusk extraction, indicating other functions for the infralabial gland besides simple food lubrication. Our results suggest a close relation between the infralabial gland's large dimensions and the seromucous nature of its secretion, and the specialization of feeding habits in *Dipsas indica*.

Supported by CAPES, FAPESP, CNPq

4.13 Comparative morphological study of venom glands of *Cryptops iheringi, Otostigmus pradoi* and *Scolopendra viridicornis* centipedes

Pedroso CM[1], Antoniazzi MM[1], Knysak I[3], Martins R[3], Guizze SPG[3], Barbaro KC[2]

[1]Laboratório de Biologia Celular, [2]Laboratório de Imunopatologia, [3]Laboratório de Artrópodes, Instituto Butantan, SP, Brazil

Introduction: Centipedes are arthropods from Class Chilopoda with a large distribution in all continents and well adapted in urban areas. For this reason, they can often cause accidents in humans, characterized by burning pain, paresthesia and edema, and sometimes developing into superficial necrosis. The venom glands of these animals are whitish and elongated, and are laterally localized inside the maxillipedes, immediately under the chitinous integument. They communicate with the exterior by a chitinous duct that ends in a pore opening subterminally in the pointed tip of each one of the maxillipedes. These constitute the stingers, resembling injection needles. **Objectives:** The aim of this study was to conduct a morphological and histochemical comparison of the venom glands of the species *Cryptops iheringi, Otostigmus pradoi* and *Scolopendra viridicornis*, representatives of the 3 genera that cause the majority of registered accidents in humans. **Methods:** The glands were dissected, fixed in Karnovsky fixative, and processed for histology in glycol methacrylate and transmission electron microscopy. PAS, Alcian blue, pH 2.5, bromophenol blue and Sudan black histochemical methods were used to characterize the chemical nature of the secretion. **Results:** The glands are externally enveloped by a layer of striated musculature, followed internally by a connective tissue layer. The glandular body is composed of elongated secretory cells arranged radially and parallel to the smaller axis of the gland. These cells are very columnar and lie individually inside an epithelial pocket, surrounded by connective tissue, followed by a monolayer of striated muscle. The secretion inside the secretory cells is observed in different stages of maturation, from small granules in the basal cytoplasm near the nuclei, to large vacuoles full of a homogeneous content, occupying most of the cellular volume. The secretion, depending on the maturation phase can show different grades of positive reaction to bromophenol blue and PAS, indicating glycoprotein content. Alcian blue and Sudan black staining are both negative. The mature content of the large vacuoles is released from the cells through perforations in the chitinous wall of the central duct, which communicates to the exterior by the subterminal pore. **Discussion:** The comparative analysis of the results indicates that the gland morphology and histochemistry of the secretion is very similar for the three species studied, except for the size of the glands, which is proportional to the size of each species, and for the gland shape, which is cylindrical in *Scolopendra viridicornis*, and polyhedral in *Cryptops iheringi* and *Otostigmus pradoi*.

Supported by FAPESP, CAPES, Fundação Butantan

4.14 The accessory gland and the primary duct of *Bothrops jararaca* snakes: morphological study

Sakai F[1], Giannotti KC[2], Carneiro SM[2], Yamanouye N[1]
[1]Laboratório de Farmacologia, [2]Laboratório de Biologia Celular, Instituto Butantan, SP, Brazil

Introduction: Venom glands of viperid snakes have a central lumen where venom is stored. Secretory cells are stimulated for a new cycle of venom synthesis after emptying the lumen either by manual extraction or biting. The accessory gland is a distinct part of the venom gland apparatus of viperid snakes, but there are few studies about accessory glands. This gland is structurally more complex than the main venom gland. However, its contribution to the composition of venom is very small. **Objective:** The aim of this study was to describe the accessory gland and primary duct of *Bothrops jararaca* during the venom production cycle through morphological analysis with light microscopy. **Methods:** *Bothrops jararaca* snakes were anesthetized with sodium pentobarbital (30 mg/kg) and the accessory glands and primary ducts from unmilked males and from females unmilked and milked 4 days prior were excised after decapitation of the snakes, fixed and embedded in Historresin. Histological sections 4 and 6 μm thick were stained with toluidine blue, Alcian blue and periodic acid-Schiff (PAS). The two last stains were used to detect acid and neutral mucopolysaccharides, respectively. **Results:** In unmilked snakes, the female accessory gland differs from that of males in their length which is about 3.5 – 4.0 mm (n=2), while in the male it is about 2.5 mm (n=2). There is no difference in the female accessory gland between unmilked and milked 4 days prior. Their structure is very similar and they possess an anterior region with narrow tubules formed by large mucous secretory cells, PAS and Alcian blue positive, and a posterior region with wide tubules lined by flattened cells with a few of them staining positive with PAS or Alcian blue. The primary duct composed of columnar cells is greatly folded, and in the gland interior it is enveloped by longitudinal tubules that originate from the anterior region of the gland. In the unmilked snakes, this duct is filled with venom, while in snakes milked 4 days prior, the primary duct is empty. **Discussion:** In the present study, we showed for the first time the morphology of the accessory gland and primary duct of viperid snakes in different stages of the venom production cycle. The presence of venom in the primary duct in unmilked snakes indicates storage of venom as in the lumen of the quiescent main gland. The accessory gland of female snakes is longer than that of male snakes, but we did not find any difference between male and female histology. The accessory gland and the primary duct of *Bothrops jararaca* are similar to those of Asiatic and African viperid snakes and to *Crotalus* species of North America.

Supported by FAPESP, Fundação Butantan

4.15 Morphological variation in the skin structure along the body of the caecilian *Siphonops annulatus* (Amphibia, Gymnophiona)

Marques-Porto R, Jared SGS, Antoniazzi MM, Jared C
Laboratório de Biologia Celular, Instituto Butantan, SP, Brazil

Introduction: Amphibians possess the most complex skin among vertebrates, having several functions such as respiration, water transport, and mechanical and chemical protection. In the suborder Gymnophiona (caecilians), the skin is also involved in adaptations to a fossorial life, such as a very smooth and lubricated skin surface and annuli that help in subterranean locomotion. It is likely that the histological structure of the integument is also adapted to this mode of life, including the epidermal arrangement as well as the distribution of the cutaneous glands. **Objective:** We aimed to describe the morphological variation in the integument structure along the body of *Siphonops annulatus* (Sa), regarding the biology of this species. **Methods:** Skin samples from different regions of the body, from snout to tail, were prepared for routine histology and electron microscopy, and histochemistry (PAS, Alcian blue, bromophenol blue and Sudan black B). **Results:** The epidermis is composed of 5 to 8 layers of epithelial cells, depending on the region of the body: thicker at the snout and ventral area; thinner at the tail end and on the back. The stratum germinativum is composed of cuboid cells that become flatter as they progress through the epidermis. The non-cornified cells of the outermost layer are somewhat flattened, while the cells of the keratinized cell layer, in the stratum corneum, are extremely flattened. The cornified layer is thicker at the snout and tail than in the rest of the body. Flask cells occur in the body epidermis, in the outer layers. In the stratum spongiosum of the dermis, as in most amphibians, two major classes of glands occur: granular glands (G) and mucous glands, of two types (MA and MB). MA are small, and have a well-defined lumen and two types of cells. They are evenly distributed throughout the body. MB, on the other hand, are more numerous at the snout, where they fill almost the whole stratum spongiosum. They become fewer at the neck and then evenly distributed throughout the rest of the body. They have a small lumen and two cell types. G glands, on the other hand, are more numerous and much larger at the rear end of Sa, where they fill most of the dermis, which is itself much deeper near and at the tail. G glands are also more numerous on the back than on the ventral part of the body. They have two cell types. One large, forming the secretory layer, filling most of the gland volume, which has no lumen, and a second type, less numerous, PAS positive, near the top of the gland and around the duct. The skin secretion at the snout is translucid, colorless and viscous, while at the rear end it is milky and fluid, in accordance with the largest occurrence of each glandular type at these same extremities. **Discussion:** The snout is rich in mucous glands, which possibly lubricate the skin as the animal plunges into the ground. At the same time, the thicker epidermis and cornified layer may confer to this region additional protection against abrasion. On the other hand, the large and abundant granular (or venom) glands in the rear end can provide defense against predators, when attacked from behind. In this way, it seems that the gland distribution along the body is closely related to the fossorial mode of life.

Supported by FAPESP, CNPq

4.16 Morphology of the femoral glands in the lizard *Ameiva ameiva* (Teiidae) and their possible role in semiochemical dispersion

Imparato BA[1], Antoniazzi MM[1], Rodrigues MT[2], Jared C[1]
[1]Laboratório de Biologia Celular, Instituto Butantan, SP, Brazil; [2]Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, SP, Brazil

Introduction: Many lizards have epidermal glands in the cloacal or femoral region with semiochemical function mainly related to sexual behavior and/or territorial demarcation. Externally, these glands are recognized as a row of pores, opening individually in the center of modified scales. They form a glandular cord or, in some species, a row of glandular beads below the dermis, and are connected to the exterior through ducts which continuously release a solid secretion. Dead cells, desquamated from the secretory epithelium, constitute the secretion, known as the "secretion plug." **Objectives:** The aim of the present work was a morphological study of femoral glands in the lizard *Ameiva ameiva*, correlated to the way the secretion is deposited in the environment. **Methods:** Skin samples of the femoral region containing the glands were removed, fixed and processed for light and electron microscopy. For light microscopy, the sections were stained with HE, toluidine blue-fuchsin and N.M.C. trichrome for a general histological study of the tissues. Other sections were subjected to special staining methods and histochemical reactions for identification of collagen and elastic fibers, general protein content, neutral and acid mucosubstances, and lipid substances. The glands were also subjected to the routine methods for transmission and scanning electron microscopy. In addition, a morphometric study of the femoral pores was conducted, using 28 specimens from the zoological collection of the Museu de Zoologia da Universidade de São Paulo. **Results:** The femoral pores in *A. ameiva* did not show significant differences between males and females. The glands are composed of germinative and secretory cells. The secretory cells pass through, at least, three stages of differentiation during which an accumulation of cytoplasm granules containing glycoprotein occurs. The cells eventually die and desquamate from the glandular epithelium, forming a friable secretory plug, mostly consisting of juxtaposed non-fragmented secretory cells. The ultrastructural study shows an abundance of granular endoplasmic reticulum confirming the protein nature of the secretion. The differentiated scales, where the glandular pores are located, are fragmented and resemble flower petals, forming a rosette. The rosette dermis is rich in collagen and elastic fibers with a peculiar nature, conferring malleability to the connective tissue. **Discussion:** The morphological analysis of the rosette-shaped scales and of the secretion plug suggests that, when the animal sits on the substrate, the femoral regions of its thighs touch the ground, pressing the malleable rosettes and cutting off tiny pieces of the secretion plugs, which are then deposited on the substrate**.** Pheromone dispersion in *A. ameiva* seems to involve a more complex mechanism than a simple breakage by chance of the plug, which is related to structural and mechanical characteristics of the rosettes and of the plugs. Although having its own peculiarities, this method of chemical signaling, similar to what has been observed in amphisbaenians, is intimately dependent on the locomotion of the animal within its territory.

Supported by Fundação Butantan, CNPq

4.17 Ultra-structure of the Duvernoy glands in Opistoglyph Xenodontinae snakes

Serapicos EO[1], Merusse JLB[2]
[1]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil; [2]Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brazil

Introduction: The non-poisonous snakes are responsible for 30% to 40% of the snakebite cases recorded by Hospital Vital Brazil in Instituto Butantan, located in São Paulo. Among them, the opistoglyph colubrid snakes have stirred interest, due to the clinical development of the accidents they have caused. **Objectives:** The aim of this study was to describe the ultra-structure of the Duvernoy glands of some species of opistoglyph xenodontinae snakes, in order to characterize the morphology of the cells that comprise them, as well as the respective secretion granules produced by these glands. **Methods:** The Duvernoy glands were collected and immediately fixed with 2.0% glutaraldehyde in 1.0 M cacodylate buffer solutions (pH 7.3). After that, they were treated with 1% osmium tetroxide and impregnated with heavy metals, for later ultra-microtomy. **Results and Discussions:** The results obtained showed that in the species analyzed, Duvernoy glands showed four types of secretion granules that were classified according to the observed electron density. The Duvernoy glands of *Philodryas olfersii* and *Philodryas patagoniensis* demonstrated very similar ultra-structural characteristics with each other. The secretory tubules displayed strongly electron-dense secretion granules, indicating that these were serous secretion granules. While the excretory ducts showed cells with variable electron-density secretion granules, showing the mucous character of these glands. In *Oxyrhopus guibei,* only medium and slightly electron-dense secretion granules were observed, as well as in *Thamnodynastes strigatus*. However, the secretory cells of each species showed their own characteristics, suggesting the occurrence of secretions with different chemical components. *Tomodon dorsatus* showed a predominance of slightly electron-dense granules, indicating the mucous nature of the secretion produced. The types of secretion granules found in the cytoplasm of the cells of Duvernoy glands from the species analyzed were shown to be associated with the serpent's diet, as well as the severity observed and described in the snakebite accidents caused by these species.

4.18 The first photographic record of defensive hemipenis display in *Micrurus* (Serpentes: Elapidae)

Serafim H[1], Duarte MR[2]

[1]Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, SP, Brazil; [2]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: Defensive tail display is widespread among most snake families, both in entirely inoffensive species, and in highly aggressive or venomous species such as coral snakes (*Micrurus*) [(1,2)]. Some coral snakes, such as a subspecies of *Micrurus frontalis* elicit defensive tail display and will protrude one or the other hemipenis when handled violently or molested [(3,4)]. **Objectives:** The aim of this study was to compare defensive hemipenis display observed in the wild in *M. frontalis* with controversial defensive tail display hypothesis reported in the literature. **Methods:** We report here on a photographic record of a hemipenis display behavior in *Micrurus frontalis* in the wild during fieldwork in Conceição do Mato Dentro, MG, southeastern Brazil (19°15´40´´ S, 43°31°58´´ W, 1,364 m altitude). **Results and Discussion:** An adult male of *Micrurus frontalis* (*ca*. 100 cm in total length) was found on 12 December 2006 at 15:00 h in rocky fields ("campo rupestre"). When unintentionally approached, the snake protruded the right hemipenis at least two minutes, an elapsed time long enough for this to be photographed. After be manipulated with a stick, the hemipenis was kept everted for another two minutes. Hemipenis eversions have been interpreted as an additional threat for predators (deimatic behavior) or as an epiphenomenon of the tail display. Based on this observed behavior of *M. frontalis* in the wild as well as on the behavior of other *Micrurus* representatives in captivity, we suspect that both hypotheses are plausible and cannot be discarded, even if it can result in a hemipenis injury by the potential predator.

1-Greene, 1973. J. of Herpetology 7: 143-161; 2-Wüster and Cox 1992. J. of Herpetology 26: 238-241); 3-Allen, 1940. Copeia: 51-52; 4- Roze 1996, Fla. Krieger Publishing Company. 328 pp.

4.19 Herpetofauna reference collection in Instituto Butantan

Calleffo MEV, Rosa JHN, Germano VJ
Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: The importance of scientific collections, besides discovering, describing and categorizing the diversity of the species, also is to make this information accessible and available, pointing out the biodiversity of this existing biological patrimony. Donations from various suppliers come from the rescue of fauna in hydroelectric area in which employees and researchers from the Institute Butantan participate and from specific collections and institutional projects. This collection is intended for taxonomic identification and classification of the catalogued species. **Objective:** The Reference Collection of the Herpetology Laboratory of the Butantan Institute was initially created for the purpose of allocating the species of amphibians and reptiles, except snakes, that arrived at the Reception of the Institute Butantan - IB. **Methods:** The sacrificed specimens are fixed in 10% formalin, later washed in running water, labeled and preserved in 70% alcohol. This reference collection is stored in a steel closet in the Casa Vital Brazil at a distance from the main collection. **Results:** At the moment, the total number of catalogued and tumbled specimens is 672: 291 lizards, 70 amphisbaenids, six (6) turtles, one (1) alligator and 304 amphibians. All the units are in the process of tumble and identification due to the new taxonomic changes. **Discussion:** The specimens destined for this collection, while still living creatures, are used to the advantage of different areas of the Butantan Institute for research, production, public exposition and educational activities. After their utilization, the specimens that are returned to the Reception - Herpetologia, are incorporated into the Reference Collection, still being organized, which researchers and students use for taxonomic reference. This Zoological Reference Collection is an annex of the "Coleção Herpetológica Alphonse Richard Hoge" of the Butantan Institute, São Paulo (IBSP-Herpeto), inserted in National Cadastre of Biological Collections of IBAMA (CCBIO). In the future, with the growth and the extension of this quantity, it is possible that it will be incorporated into the main collection of the Butantan where it will be available to the outside public.

4.20 Ophidian fauna study in a delimited area of Serra da Cantareira

Travaglia-Cardoso SR[1], Albuquerque CC[1], Puorto G[2]
[1]Laboratório de Herpetologia, [2]Museu Biológico, Instituto Butantan, SP, Brazil

Introduction: The State Park of Serra da Cantareira is the largest urban forest of the world, with altitudes varying from 860 to 1,215m. Located in the Atlantic Plateau, between the Maciço da Cantareira and Serra de Piracaia, it occupies areas of four districts: São Paulo, Caieiras, Mairiporã and Guarulhos, totaling 7.9 hectares. This area of the Atlantic Forest, rich in biodiversity, extends much further beyond the Park's boundaries, in particular contiguous reserves, so that it turns the whole area of Cantareira into an important refuge for the preservation of species in danger of extinction. As the area was occupied by farms for tea, coffee and sugarcane until the end of the XIX century, most of the forest that covers the Serra today is regenerated. It was not until about 1890 that the government of São Paulo realized the importance of that area as source for the water supply of the city and passed the first laws of environmental protection. Towards the boundary of Mairiporã with the district of Caieiras, we encounter one of the most preserved sites of Serra da Cantareira: Sausalito. A quarter of its almost 3 million square meters corresponds to green areas that are located on an uneven relief, with a mean altitude of 1,200 m. As a great deal of these areas are next to hillsides, deep valleys and legal reserves of permanent protection, Sausalito became a lake-crowded place, where the presence of wild animals has been recorded. To keep that natural patrimony, the residents of Sausalito founded the Olho D´Água Institute of Environmental Education, Research and Conservation of the Biodiversity of the Atlantic Forest. Through the community's involvement in environmental projects and in partnerships with research entities, that Institute has been promoting the study of biodiversity and helping its maintenance. Starting with classes provided by Instituto Butantan (IB) researchers for employees and residents, an invitation resulted to conduct a study about the ophidian fauna present in Sausalito. **Objectives and Methods:** This work presents the study of the ophidian fauna currently found in Sausalito. It was accomplished through the collection conducted by the investigators of IB and also through the photographic recordings and collections carried out by residents and employees of the condominium in the last three years. Some specimens are now included in the Herpetological Collection of IB. **Results and Discussion:** There were 16 species of snakes identified: two that belong to the family Viperidae (*Bothrops jararaca*, *Bothrops jararacussu*), one to the family Elapidae (*Micrurus corallinus*) and 13 species to the family Colubridae (*Chironius bicarinatus*, *Echinanthera undulata*, *Erythrolamprus aesculapii*, *Helicops modestus*, *Liophis miliaris*, *Liophis typhlus*, *Oxyrhopus guibei*, *Philodryas olfersii*, *Philodryas patagoniensis*, *Sibynomorphus mikanii*, *Spilotes pullatus*, *Tomodon dorsatus*, *Xenodon neuwiedii*); and other reptiles: an amphisbaenid (*Amphisbaena alba*) and a legless lizard (*Ophiodes fragilis*). In spite of the area studied being in a moderately populated region, the results show satisfactory nature conservation, with the representation of a good part of the snakes inhabiting areas of the Atlantic Forest.

Acknowledgements: D. P. Ramos and M. M. T. Rocha for the collaboration in the collecting.

4.21 The non-Amazonian species of *Phoneutria* Perty, 1833 (Araneae, Ctenidae) with description of a new species

Martins R[1], Bertani R[2]
[1]Laboratório de Artrópodes; [2]Instituto Butantan, SP, Brazil

Introduction: Species of the genus *Phoneutria* Perty are known as "aranha-armadeira" in Brazil, or armed spider, Brazilian wandering spider or banana-spider in English speaking countries. They are medium to large-sized spiders, reaching from 17 to 48 mm in total body length and 180 mm with stretched legs. This genus is responsible for a large number of spider bites in Brazil, being the main agent in most areas. The venom has a neurotoxic action and many investigations have been carried out on its components and the epidemiology of bites. **Objectives:** Using the morphology of male and female genitalia and color pattern, we reviewed taxonomically the non-Amazonian species of *Phoneutria*. **Methods**: We examined specimens of the institutions: IBSP, Instituto Butantan, São Paulo; MZSP, Museu de Zoologia da Universidade de São Paulo, São Paulo; UNB, Universidade Federal, Brasília. Drawings were prepared with a stereoscope microscope NIKON SMZ 1500 and the aid of a drawing tube. For scanning electron micrographs we used a JEOL JSM 840A scanning electron microscope (LME-IFUSP). **Results and Discussion**: The review of the taxonomy of the non-Amazonian species of *Phoneutria*,resulted in five species. We revalidate two species currently placed in synonym with *P. nigriventer* and describe one new species from Brazil – *P. eickstedtae*. *Phoneutria keyserlingi* (Pickard-Cambridge) and *Phoneutria pertyi* (Pickard-Cambridge) are considered valid and redescriptions of male and female *P. pertyi* are presented. *P. luederwaldti* Mello-Leitão is considered *nomen dubium*. A map is presented showed the distribution of the non-Amazonian species among the different states of Brazil: *P. bahiensis* is distributed in southern Bahia to northern Espirito Santo; *P. keyserlingi* from southern Rio de Janeiro to southern Santa Catarina, in the coastal region; *P. pertyi* in extreme southeast of Bahia, eastern Minas Gerais, Espirito Santo and northwestern Rio de Janeiro; *P. nigriventer* in Minas Gerais, Goiás, Mato Grosso do Sul, Rio de Janeiro, São Paulo, Paraná, Santa Catarina and Rio Grande do Sul; and the *P. eickstedtae* sp. nov. in southern Tocantins, Goiás, Mato Grosso and Mato Grosso do Sul.

Supported by FAPESP

4.22 On the genus *Lasiodora* C. L. Koch 1850 (Araneae, Theraphosidae)

Bertani R
Instituto Butantan, SP, Brazil

Introduction: The genus *Lasiodora* comprises the largest spiders of the Brazilian Atlantic Rainforest and have been known to naturalists at least since 1641 when the Dutchman Albert Eckhout illustrated one specimen in Pernambuco, Brazil. It is one of the older theraphosid genera, being derived from the division of the genus *Mygale* and included formerly six species. Ausserer, Bertkau, Thorell, Simon and Pocock described several new species, and in 1901 Pocock chose as type species *L. klugi*, from Bahia, Brazil and transferred several species to other new genera proposed by him. Other authors such as Strand, Chamberlim and Mello-Leitão contributed by describing many new species. After the synonymy of *Crypsidromus* with *Lasiodora* in 1996, the genus now has 38 species and one subspecies, the majority described for Brazil (24). The genus is close to *Vitalius*, *Nhandu* and *Proshapalopus*, differing by the presence of stridulatory bristles on the prolateral coxae of legs I-IV. **Objectives:** The aim of this study was to revise the genus *Lasiodora* C. L. Koch. **Methods:** A comparative morphological analysis was performed. **Results and Discussion:** In this study, six species were considered valid, all them were distributed only in Brazil: *L. isabellina* (synonyms: *L. benedeni* Bertkau, *L. curtior* Chamberlin, *L. differens* Chamberlin, *L. cristata* Mello-Leitão, *L. difficilis* Mello-Leitão and *L. mariannae* Mello-Leitão), *L. itabunae* Mello-Leitão, *L. subcanens* Mello-Leitão, *L. parahybana* Mello-Leitão and *L. klugi* C. L. Koch. A new species was detected and is described. *L. lakoi* Mello-Leitão belongs to the genus *Megaphobema* and *L. spinipes* Ausserer to *Theraphosa*. *L. sternalis* Mello-Leitão is a synonym of *Acanthoscurria gomesiana* Mello-Leitão. The following species are considered "nomina dubia," since the types could not be located and the descriptions were insufficient for allowing identification: *L. acanthognatha* Mello-Leitão, *L. boliviana* (Simon), *L. citharacantha* Mello-Leitão, *L. cryptostigma* Mello-Leitão, *L. dolichosterna* Mello-Leitão, *L. dulcicola* Mello-Leitão, *L. erythrocythara* Mello-Leitão, *L. fallax* (Bertkau), *L. fracta* Mello-Leitão, *L. moreni* (Holmberg), *L. pantherina* (keyserling), *L. pleoplectra* Mello-Leitão, *L. saeva* (Walckenaer) and *L. striatipes* (Ausserer). Species from Central America and Venezuela will be transferred to other genera, mainly to *Hapalopus* Ausserer.

Supported by FAPESP

4.23 Evolution of sperm transfer behavior in Arachnida

Silveira MC[1,2], Japyassú HF[2]
[1]Instituto de Psicologia, Universidade de São Paulo, SP, Brazil; [2]Laboratório de Artrópodes, Instituto Butantan, SP, Brazil

Introduction: The group of arachnids is composed of eleven extant orders: Araneae, Opiliones, Scorpiones, Pseudoscorpiones, Palpigradi, Amblypygi, Uropygi, Schizomida, Ricinulei, Acari, and Solifugae. Mating in Araneae, Opiliones, and Ricinulei occurs by direct sperm transfer from male to female through a specialized organ. In Scorpiones, Pseudoscorpiones, Schizomida, Amblypygi, and Uropygi, mating occurs indirectly through a spermatophore. The mating behavior in these orders consists in an elaborate courtship and deposition of a pedunculated spermatophore in a suitable substrate. During courtship, the male often grasps the female's body and leads it to the spermatophore. **Methods**: In this study, we optimized those behaviors in the phylogeny of Arachnida, and discussed the evolution of sperm transfer. **Results and Discussion**: We found three distinct evolutionary origins for mating with a spermatophore preceded by courtship. One of these origins reveals a homology to this character in orders Amblypygi, Uropygi, and Schizomida. The use of spermatophore with courtship in Scorpiones and Pseudoscorpiones is convergent, contrasting assumptions of homology to this behavior in these orders. On the other hand, it agrees with the literature that indicate that this behavior is absent in early pseudoscorpions and it is a synapomorphic trait to most derived species. Our results also agree with authors who demonstrate that the use of a spermatophore evolved several times among Arthropods.

Supported by CNPq

4.24 Review and cladistic analysis of the Neotropical tarantula genus *Ephebopus* Simon (Araneae, Theraphosidae) with notes on the Avicullariinae

West RC[1], Marshall SD[2], Fukushima CS[3,4], Bertani R[4]
[1]3436 Blue Sky Place, Victoria, BC, Canada; [2]Department of Biological Sciences, Northwestern State University, Natchitoches, LA, USA; Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, SP, Brazil; [4]Instituto Butantan, SP, Brazil

Introduction: No other theraphosid genus has had its taxonomic position as uncertain as *Ephebopus* Simon, 1892. Formerly included in the group Selenocosmieae (1892), the genus was later transferred to such different groups as Avicularieae (1901), Phoneyuseae (1903), back to Avicullariinae (1923), Eumenophorinae (1942), Theraphosinae (1985), and again back to Avicullariinae (1991). Thus, it was classified in four of the eight theraphosid subfamilies. The genus is generally fossorial, however, *Ephebopus murinus* has a developmental stage that is arboreal. *Ephebopus* has as synapomorphy the presence of a pad of urticating hairs type V on the prolateral distal area of the pedipalps, a feature unique in spiders. The genus is distributed in northeastern South America: Brazil, Guyana, Suriname, and French Guiana. **Objectives:** The aim of this study was the review and cladistic analysis of the genus *Ephebopus*. **Methods:** A cladistic analysis with 21 taxa and 57 characters was performed and included representatives of seven of the eight theraphosid subfamilies. The cladistic software packages X-Pee-Wee, Nona, Hennig86 with equal weights and successive weights were used. **Results:** The analysis resulted in one, three, eleven and nine trees, respectively. The genus comprises *E. murinus*, *E. uatuman*, *E. cyanognathus*, *E. rufescens* and a new species from Guyana. *Ephebopus violaceus* Mello-Leitão, 1930 shall be transferred to *Tapinauchenius violaceus* (Mello-Leitão, 1930) and is considered a senior synonym of *Tapinauchenius purpureus* Schmidt, 1995. *Ephebopus fossor* Pocock, 1903 is considered "nomem dubium." **Discussion:** The preferred tree obtained was with X-Pee-Wee and showed that Avicullariinae shall be monophyletic and include *Avicularia* Lamarck, *Iridopelma* Pocock, *Pachistopelma* Pocock, *Tapinauchenius* Ausserer, *Psalmopoeus* Pocock, *Ephebopus*, *Stromatopelma* Karsch and *Heteroscodra* Pocock, having as a synapomorphy the well-developed scopulae on tarsus and metatarsus I-II. The genus *Ephebopus* is basal in the cladogram of Avicullariinae which helps explain its controversial taxonomic position.

Supported by FAPESP, CAPES

4.25 The genus *Pamphobeteus* Pocock, 1901 (Araneae, Theraphosidae) in Brazil with a description of two new species and a different stridulatory organ

Bertani R[1], Fukushima CS[1,2], Silva Jr PI[3]
[1]Instituto Butantan, SP, Brazil; [2]Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, SP, Brazil; [3]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, Instituto Butantan, SP, Brazil

Introduction: The genus *Pamphobeteus* was described by Pocock in 1901 who distinguished it from *Lasiodora* C.L. Koch by the absence of both a scopula on the inferior side of the femur of the first leg and stridulatory organ on the anterior side of the coxa of the first leg and on posterior side of the palpal coxa. In 1903, Pocock described three new species and also transferred some species from *Lasiodora* to *Pamphobeteus*. Many more species were described for this genus by different authors since then, mainly by Mello-Leitão, Piza and Soares who described many species for Brazil. All these Brazilian species of *Pamphobeteus* were later transferred by Lucas, Silva Júnior & Bertani to the new genus *Vitalius* erected by these same authors or to *Lasiodora*, *Eupalaestrus* and *Proshapalopus*. The ten species nowadays accepted in the genus (*P. antinous, P. augusti, P. ferox, P. fortis, P. insignis, P. nigricolor, P. ornatus, P. petersi, P. ultramarinus* and *P. vespertinus*) are distributed in northwestern South America. The genus is more specious in Colombia and Ecuador, in the Andes mountain range, and no species of *Pamphobeteus* has yet been recorded for Brazil. **Objectives**: The aim of this study was to investigate the *Pamphobeteus* species in Brazil. **Methods:** A comparative morphological analysis was performed. **Results**: We described two new *Pamphobeteus* species from northwestern Brazil (states of Acre, Rondônia, Mato Grosso and Amazonas) and a new record for *P. nigricolor* in the state of Amazonas was established. **Discussion**: The new species are closely related to *P. antinous* due to the wide concave/convex embolus shape and the huge size of the specimens. A stridulatory organ consisting of spiniform setae was observed in all coxae of legs and stridulation of the hind legs was recorded, videotaped and described.

Supported by FAPESP, CAPES

4.26 The identity of *Avicularia brunnipes* C. L. Koch (Araneae, Theraphosidae, Theraphosinae) with the redescription of the species and the description of a new genus

Fukushima CS[1,2], Nagahama RH[1,2], Bertani R[2]
[1]Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brazil; [2]Instituto Butantan, SP, Brazil

Introduction: Theraphosinae is a specious New World subfamily having almost half of the 900 theraphosid species so far described. The taxonomy of the group is complex and there is a lack of revisions for most genera. The result is that many of the species described mainly in the XIX century cannot be identified. The lack of specimen sampling is another reason why the identity of these species remains a mystery. Most of them were collected in expeditions carried out by naturalists more than a century ago and were never recollected again. The genus *Avicularia* Lamarck is a typical case: there are 29 species considered as "nomina dubia," all described between 1805-1889. **Objectives**: The aim of this study was to consider the description of a new genus for this specimen. **Methods**: Recently, a collection was made in the northeastern Brazilian Atlantic Forest of specimens of a small theraphosine resembling an old species described originally as *Mygale brunnipes* C. L. Koch and now placed in *Avicularia* genus as "nomem dubium." C. L. Koch's figure presents a very characteristic coloration, which was found in the individuals that were collected in the states of Paraíba, Alagoas and Pernambuco, Brazil. The structures were analyzed and submitted for cladistic analysis. **Results**: The species resembles *Plesiopelma* and *Homoeomma* by the shape of the genitalia, but differs from *Plesiopelma* by lacking the metatarsus I protuberance in males and from *Homoeomma* by lacking the digital apophysis on the male palpal bulb. Furthermore, contrary to these two genera that have a metatarsus I folding between the two branches of the tibial apophysis, in this species the metatarsus I folds against the retrolateral branch tip. **Discussion**: A preliminary cladistic analysis including *Avicularia brunnipes*, *Plesiopelma* spp., *Homoeomma* spp., *Cyriocosmus* spp., *Hapalopus* spp., *Tmesiphantes* sp., *Euathlus* sp. and *Grammostola* sp. shows that the species should be included in a new genus.

Acknowledgements: Reserva Biológica de Saltinho, Estação Ecológica de Murici, IBAMA
Supported by FAPESP, CAPES

4.27 Mating behavior in the spermatheca-lacking species *Sickius longibulbi* Soares & Camargo (Araneae, Theraphosidae)

Bertani R[1], Fukushima CS[1,2], Silva Jr PI[3]
[1]Instituto Butantan, SP, Brazil; [2]Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, SP, Brazil; [3]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, Instituto Butantan, SP, Brazil

Introduction: The family Theraphosidae is a predominantly New World taxon, with a poorly elucidated biology. Nevertheless, theraphosid reproductive behavior pattern is very similar, concerning the courtship, clasping and palpal insertions. Most times, mating occurs with the male positioned in front of the female, which raises the body, allowing the male to reach the genital opening. However, the mating of *Sickius longibulbi* (the first Mygalomorphae described with no spermathecae) is clearly different from the theraphosid pattern. **Objectives**: The aim of this study was to examine the sexual behavior of *Sickius longibulbi* Soares & Camargo, a small Brazilian theraphosid. **Methods**: The mating behavior of nine pairs of *S. longibulbi* was videotaped and analyzed. **Results**: In this species, the male showed almost no courtship of the female. There was also a high degree of aggressiveness involved in this mating. As the female did not present herself passively, the male bit her several times. Despite male aggressiveness, there was no record of cannibalism. Moreover, an extraordinary behavior was detected: the male tried to knock the female down, pulling her legs III or pulling her entire body until the female's dorsal cephalothorax rested against the substratum. After this fall, the male positions himself at an angle of 90^{o} degrees relative to the female, sustained by his palpal bulbs, which were inserted into the female genital opening, with his tibial apophysis clasped in the female's fangs. **Discussion**: These movements seem to demand much energy. It is not possible to know whether the insemination occurs only in this position. However, we suggest that males that copulate in this position are more successful than others, since its extremely long palpal embolus would deposit more sperm deeply in the female oviducts. In *Encyocratella olivacea* Strand, the other mygalomorph species known to lack spermathecae, the mating behavior is typical for theraphosids. Thus, the lack of spermathecae cannot explain alone the distinct mating behavior of *Sickius longibulbi*.

Supported by FAPESP, CAPES

4.28 *Loxosceles intermedia* (Araneae, Sicariidae) in wild environment in Paraná State, Brazil

Gonçalves-de-Andrade RM, Shoji RF, Souza-Santos P, Melo HLAS, Tambourgi DV
Laboratório de Imunoquímica, Instituto Butantan, SP, Brazil

Introduction: The *Loxosceles* genus includes spider species of public health importance, given that their poison induces severe local and/or systemic reactions. A hundred species have been described to date for the whole of the Neotropical region, Europe, Asia and Africa, with ten being present and/or endemic in Brazil. Three of these, *L. gaucho, L. intermedia and L. laeta* are Loxoscelism agents especially in the southwestern and southern regions of the country. Small, with slender long legs, six eyes displayed in three dyads and brown in color, they display preferably nocturnal habits. Their natural biotope is mainly tree hollows, steep wall slits, dry leaves, rocky walls and caves. There are extensive studies of envenomation caused by synanthropic species of *Loxosceles*, but studies are scare regarding their systematics, biology and ecology. Among the ecotopes where the *Loxosceles* have been found, the karst stands out. They are the most representative troglophile arachnidian in Brazil. The Brazilian Registry of Caves comprises 3,990, but there is still a vast number of Brazilian caves to be registered or described. **Objectives**: The aim of this study was the identification of the *Loxosceles* population in the exuberant karstic area on the grounds of its significant presence in this ecotope. **Methods:** The spiders were captured manually in October, 2006 in the Parque Estadual Vila Velha (3,122.11 hectares), Ponta Grossa municipality, Paraná State, Brazil (IBAMA 156/2006, IAP 39/06). Vila Velha names the grouping of natural sculptures of ruin shaped aspect developed in basically red colored periglacial arenites of the Upper Carboniferous with well preserved ecosystems in the area of Campos Gerais. The cementation by iron and manganese oxide is dominant and determinant of the shapes and colors that constitute the natural sculptures. The superficial draining of the rain water results in mechanical erosion, dissolution and reprecipitation. It forms wrinkles in the rocky walls that constitute the wide topped towers and promotes alveolar erosion. The ruin shaped formation of Trilha dos Bosques dos Arenitos and the building material shelter, the rocky sedimentary collapse wells and its adjacent wood of Furnas were explored. **Results and Discussion:** A population of *Loxosceles* was identified in both study sites. The collected specimens were identified by morphological characters as *L. intermedia* – the first recording in a natural environment in Paraná State. On the Trilha dos Arenitos the specimens were found in the natural sculptures of ruin shaped formations containing vertical fractures – adequate substrate for the nesting of this spider. In this site especially in the arenitic alveolus, vestiges such as exuviae and webs with food residues were visualized. In Furnas the *Loxosceles* was not found in the wood but showed markedly dense populations in the building material shelter. The presence of *L. intermedia* in a natural environment in Paraná State proves the endemism of this species for the region. On visualizing the arenitic formations with its alveolus inhabited by the brown spider it was possible to realize that by accumulating debris, bricks and tiles man mimics this arachnidian's natural habitat promoting its synanthropization.

Supported by FAPESP

4.29 Is reeling a plesiomorphic predatory behavior in Araneoidea orbweavers?

Gonçalves VP, Garcia CRM, Japyassú HF
Laboratório de Artrópodes, Instituto Butantan, SP, Brazil

Introduction: Reeling is a predatory behavior originally described among spiders of the families Theridiidae and Pholcidae. In this work, we show the occurrence of reeling in basal families of orbiculariae group (Uloboridae, Araneidae and Tetragnathidae), after specific web manipulations. **Objectives**: The main objective of this work was to analyze the evolution of reeling among orbiculariae spiders. **Methods**: The orbwebs were manipulated in nature (Araneidae and Tetragnathidae) and in the laboratory (Uloboridae), and the frequency and intensity of reeling in these conditions were compared to results previously obtained with theridiids in the laboratory. Since the degree of hunger could theoretically affect spiders' predatory behavior, and since this factor cannot be controlled outside the lab, we planned a first experiment in order to make sure that the predatory behavior of nature and lab spiders was comparable. **Results and Discussion**: The results of this first experiment show that the degree of hunger does not influence the frequency and timing of reeling in *Zosis geniculata* (Uloboridae). In order to understand the evolution of reeling among orbiculariea spiders we collected data from this behavior in 12 species [*Z. geniculata* (Uloboridae), *Micratena nigrichelis*, *Alpaida veniliae*, *Metazygia rogenhoferi*, *M. gregalis* (Araneidae), *Leucauge* sp1 and *Leucauge* sp2 (Tetragnathidae), *Latrodectus geometricus*, *Theridion evexum*, *Achaearaneae tepidariorum*, *A. cinnabarina* and *A. digitus* (Theridiidae)], totaling 240 individuals. Reeling occurred in all species analyzed. Nevertheless, the frequency and average intensity of reeling in each species, when optimized in the phylogeny of the group (Mesquite program), do not show a clear evolutionary signal: there are many oscillations in the level of these measures. Besides this, we observed that from Uloboridae to Theridiidae there is a progressive specialization in the predatory behavior. Thus, although reeling seems to be homologous in every spider from the orbiculariae group, a fine-grained analysis of spider's performance shows that in the basal families this behavior is still much under the control of environmental conditions (such as the actual characteristics of each web), and that these external conditions evolved gradually in such a way (appearance of gumfoots, for example) as to make this reeling behavior a specialized, yes or no response.

4.30 Grooming in Mygalomorphae

Huffenbaecher C[1,2], Japyassú HF[1,2]
[1]Instituto de Psicologia, Universidade de São Paulo, SP, Brazil; [2]Laboratório de Artrópodes, Instituto Butantan, SP, Brazil

Introduction: Grooming is a series of behaviors directed to an animal's own body and present in the whole animal kingdom, usually quite stereotyped, consisting of somewhat fixed patterns of action. There are indications that it is not a learned behavior. In insects, grooming is defined as the stereotyped acts that involve scratching the legs against each other and the body. Studies about grooming in flies show a high degree of stereotypy, suggesting its use to detect phylogenetic relationships even between distant species. There are minor differences in grooming patterns among closely related species, differences that are usually related to simple evolutionary losses or gains of behavioral acts. Grooming has not yet been described in Araneae and seems to be especially important among mygalomorphs. These spiders are rather morphologically homogeneous, and there is a relative difficulty in finding characters for the reconstruction of its phylogeny. **Objectives:** In this study, we looked for grooming behavior as a new source of characters in the group. **Methods:** We built an ethogram for grooming in 2 Theraphosidae genera (*Acanthoscurria* and *Vitalius*). These spiders were in a room with the light-dark cycle reversed. We observed 30 grooming bursts in 15 specimens; also observed spiders from other Mygalomorphae families, which had fossorial habits (Barychelidae, Idiopidae, Actinopodidae and Nemesiidae); glass tubes were introduced in the boxes, and they adopted them as their nests. The observations were made by scanning, with all spiders observed simultaneously; when one specimen started grooming, the observation was directed toward that one. **Results**: Long grooming bursts were recorded, ranging from 10 to 180 min, with 98 to 201 transitions of categories in each burst. Although grooming, as depicted from studies in other taxa, is more frequent when the animals are more active, in our observations grooming seemed to be more common on rainy days and after feeding the spiders with neonate mice. After almost 70 h of observations, we built a preliminary ethogram describing 11 categories for the 2 Theraphosidae genera. In spite of these time-consuming scannings, and after an additional full 24 h recording, only 2 bursts were observed on the fossorial species (Barychelidae and Actinopodidae). Grooming follows essentially the same sequential pattern in both Theraphosidae genera: first the spider cleans one side of the body, starting with the anterior legs, continuing through the abdomen and ending in the spinnerets; this sequence is repeated on the other side of the body. Palps and fangs alternate up and down movements throughout the burst. **Discussion:** The ethogram still does not allow a quantitative or qualitative differentiation between the genera. This description of grooming in Mygalomorphae is part of a wider research aimed at understanding the evolution of this dataset at the family level. In addition to the comparative analysis, this work intends to test new sources of characters for the reconstruction of the evolutionary history of this group. Grooming behavior seems promising in this sense due its high stereotypy and relative context independence, characteristics that allow the phylogenetic reconstruction even in distant taxa, and that favor the preservation of phylogenetic signals even in ancient groups, such as mygalomorph spiders.

Supported by CAPES

4.31 Subsociality in *Latrodectus* Walckenaer, 1805 (Araneae, Theridiidae)?

Bertani R[1], Fukushima CS[1,3], Martins R[2]
[1]Instituto Butantan, SP, Brazil; [2]Laboratório de Artrópodes, Instituto Butantan, SP, Brazil; [3]Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, SP, Brazil

Introduction: Subsociality consists of extended maternal care, mutual tolerance and cooperation between individuals within a colony. It is rare in spiders and it has been proposed to be a necessary step towards sociality. Scattered in spiders, subsociality is concentrated in the family Theridiidae, occurring in species of four genera: *Achaearanea* Strand, *Anelosimus* Simon, *Theridion* Walckenaer and *Helvibis* Keyserling. Recent cladistic analysis suggests that this behavior evolved independently in each of these genera, all belonging to the "lost colulus + *Anelosimus* clade." It was also predicted that additional subsocial species would be discovered inside this clade. **Objectives**: The aim of this study was to report on an additional case of subsociality for the genus *Latrodectus.* **Methods**: Specimens of the *Latrodectus* were observed in the Serra do Cipó, Minas Gerais, Brazil. In a road bank, a web of *Latrodectus* cf. *curacaviensis* with 1 x 0.3 meters was found, harboring roughly twenty individuals. The spiders were photographed, collected and kept in the laboratory. There, it was offered adult *Tenebrio molitor* beetles for observations of behavior. **Results:** We observed in the left upper web corner an old eggsac with several small skin sheddings. In the right center margin, an adult beetle was found, being eaten at the same time by six spiders. Among them there were some subadult males. In another part of the web there was another prey being wrapped by a subadult male and a small female. In laboratory spiders, after touching the sticky "gumfoot" line of the web, the beetle was attacked by a female that wrapped it. The other individuals were then attracted by the vibrations of the web and began to feed on the beetle. No aggression was seen by the individuals. The behavior was repeated again after the introduction of additional beetles. **Discussion:** The subsociality behavior described here is the first record for the genus *Latrodectus* and also for a theridiid species out of the "*Anelosimus* + lost colulus" clade. As subsociality evolved independently several times, it was not surprising to find it occurring again, even though out of the predicted clade.

Supported by FAPESP, CAPES

4.32 The first record for *Wolbachia* in a scorpion: the parthenogenetic yellow scorpion *Tityus serrulatus* (Scorpiones, Buthidae)

Suesdek-Rocha L[1], Bertani R[2], Silva Jr PI[3], Selivon D[4]

[1]Laboratório de Parasitologia, [2]Instituto Butantan, [3]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, Instituto Butantan, SP, Brazil; [4]Departamento de Biologia, Instituto Biociências, Universidade de São Paulo, SP, Brazil

Introduction. *Wolbachia* is an intracellular endosymbiont, alpha-proteobacteria, of hundreds of arthropods and nematodes. They are capable of manipulating the reproduction of arthropod hosts, inducing parthenogenesis, selectively killing males and inducing feminization of genetic males. Among arthropods, *Wolbachia* has been isolated from insects, crustaceans and mites, and more recently, it was also detected in spiders and pseudoscorpions. Here we report for the first time the presence of *Wolbachia* in a scorpion, the yellow parthenogenetic scorpion *Tityus serrulatus* Lutz & Mello, which is the most medically important species in South America. **Objectives**: The aim of the study was to test the hypothesis of the presence of *Wolbachia* in the yellow parthenogenetic scorpion *Tityus serrulatus*. **Methods**: Total DNA was extracted from 10 individuals of *T. serrulatus* (three adults and seven juveniles) and was submitted to PCR for detection of W-Spec fragment of *Wolbachia*16S rDNA. PCR-amplified fragments were sequenced using BigDye 2.0 automated sequencing kit with the same W-Spec primers. Sequences were obtained from an ABI377 sequencer and were analyzed using the software Sequence Navigator and compared with sequences from the GenBank database. A fragment corresponding to the W-Spec was amplified in all samples. **Results**: Its resulting sequence of 414 bp was deposited in GenBank. Sequences were identical in all 10 samples and were also 100 % similar to other 16S rDNA sequences, such as those from *Wolbachia* endosymbiont of *Drosophila innubila*, and the Hymenopterans *Nasonia longicornis* and *Nasonia giraulti*, therefore confirming *T. serrulatus* to be a host. **Discussion**: This appears to be the first record of *Wolbachia* in scorpions according to the updated online database of *Wolbachia* hosts. The present new record of *Wolbachia* opens a new field for the study of parthenogenesis in scorpions. One of the questions to be answered is the possible influence of the endosymbiont in parthenogenesis. *Wolbachia* has been suspected to cause sexual malformations in bothriurids, which straightens this idea. The possible influence could be accomplished by determining the effects of antibiotic-mediated bacteria elimination in the scorpion, as has been done in the wasp *Trichogramma*. Such experiments are currently being carried out by the authors.

Supported by FAPESP

4.33 Study of dispersion and overlapping of the *Tityus serrulatus* Lutz & Mello, 1922 and *Tityus bahiensis* (Perty, 1833) (Scorpiones, Buthidae) between 2000 and 2005 in the State of São Paulo, Brazil

Candido DM[1], Barbaro KC[2]
[1]Laboratório de Artrópodes, [2]Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil

Introduction: In the decades of 1940, 1950 and 1960, the data on scorpionism in Brazil was restricted to some cities such as São Paulo, Ribeirão Preto and Belo Horizonte. *Tityus serrulatus* and *Tityus bahiensis* are responsible for most of the reported accidents in the country. The most severe cases of scorpion envenomation are caused by *T. serrulatus*. After the implementation of a notification system for accidents by venomous animals in the country (1988), a significant increase in the number of accidents was reported. In the last years, the distribution of these animals expanded, including the urban areas of the state of S. Paulo. **Objectives:** The aim of this work was to estimate the dispersion of *T. serrulatus* and its dominance over *T. bahiensis* habitats in S. Paulo. **Methods:** Dispersion data was compiled from the reception of the Laboratory of Arthropods and Center of Monitoring Epidemiologist of São Paulo (2000-2005). **Results:** In the year 2000, out of 645 cities of the state only 166 reported the occurrence of the two species, yet in 2005 it increased to 415, representing a 150% increase. The total number of cities for *T. bahiensis* rose from 141 to 288, representing a 104% increase. *T. serrulatus* rose from 110 to 352 cities, representing a 220% increase. Isolated data by species indicate that in 2000, 55 cities showed only *T. bahiensis* and 66 only *T. serrulatus*. In 2005, *T. bahiensis* was recorded in 51 cities, while *T. serrulatus* appeared in 113, representing a decline of 7% for *T. bahiensis* and increase of 71% for *T. serrulatus*. A detailed analysis of the two species overlapping showed that in the period studied: A) *T. bahiensis* was recorded before *T. serrulatus* in 66 cities, and of these, after five years, 35 had both species occurring together, 20 indicated only *T. serrulatus* and 10 cities had *T. bahiensis*; B) *T. serrulatus* was recorded before *T. bahiensis* in 79 cities, of these, in the end, 36 cities had both species occurring together, 27 had continued only with *T. serrulatus* and 16 with *T. bahiensis*; C) 48 cities had both species in 2000, and after five years, *T. serrulatus* was found in 39 and *T. bahiensis* only in 31 cities, representing a decline of 19% for *T. serrulatus* and 35% for *T. bahiensis*; D) *T. serrulatus* stayed alone in 13 of the 39 cities, while *T. bahiensis* stayed alone only in 5 of the 31 cities. **Discussion:** The data analyzed show the great capacity of adaptation of *T. serrulatus*, a species that was introduced into the state. Parthenogenesis reproduction of *T. serrulatus* also contributes to this expansion and settlement. The data also show the dominance of this species over *T. bahiensis*. In cities where initially the two species occurred together, at the end of the study, *T. serrulatus* remained in a larger number of cities compared to *T. bahiensis*. *T. bahiensis* is a native species of São Paulo, but the destruction of its natural environment, favors its dispersion, although to a lesser extent than *T. serrulatus*, emphasizing again the dominance of this species. Areas demonstrating sanitation problems and garbage accumulation, also favor the adaptation of these animals, which feed basically on insects.

5. Animal care and veterinary diseases

5.01 Phaco aspiration in snakes

Albuquerque CE[1,4], Guedes PM[2], Barros PSM[3]
[1]Laboratório de Odontologia Comparada, [3]Departamento de Cirurgia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Brazil; [2]Veterinário Autônomo, SP, Brazil; [4]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: Phacoemulsification surgery is routine practice in veterinary ophthalmology of pets, and is considered similar for human beings. In the clinical and surgical treatment of snakes, cataract patients do not benefit from any of the surgical techniques available. This is the first report describing a successful technique in serpents. In snakes, the upper and lower lids fuse together to form a structure known as the spectacle, which is vascularized, transparent, rigid, and renewed in each ecdysis (skin shedding). The tear film fills the space between the spectacle and the cornea. The spectacle represents a barrier for drugs to reach the eye ball, being a limiting factor in ophthalmologic treatments. **Methods:** We operated only one eye of five specimens of *Chironius bicarinatus*. This species was chosen for being a diurnal and arboreal colubrid snake, highly dependent on its sharp vision. The presence of the spectacle complicates the access to the eye. However, this structure does not re-grow during ecdysis if removed, even if a pedicle is maintained for nourishment. Thus, it cannot be partially or totally removed as the lack of tears is a limiting factor in ocular maintenance. The way to perform the surgery was to open a 4X2 mm window on spectacle to access the eye. The entrance to the anterior chamber was performed using a 3.2 mm angled scalpel, although we made a 2-mm incision. The next steps were: filling the anterior chamber with viscoelastic substance and capsulotomy performed with an insulin needle. The tearing from external layers of crystallin was removed by aspiration with a syringe and fine caliber aspiration needle with Ringer's lactate solution until only the nuclei remained. Due to the highly dense characteristics of the nuclei, the corneal incision has to be enlarged by approximately 2 mm to expel the crystallin. The cornea was sutured using nylon 10-0. The spectacle window was not sutured because of its rigidity and to allow medication to reach the eye during the post-operative period. The post-operative treatment consisted of antibiotic and steroidal anti-inflammatory eye drops (Tobradex©) TID, for 45 days or until the first ecdysis. During the recovery period, the animals were kept in a warm room with a controlled environment, in plastic boxes lined with cardboard paper, stones, tree branches and water *ad libitum*. **Results:** During the ecdysis process, we noticed a corneal callosity at the spectacle incision site, which persisted after some skin sheddings. No apparent stereotypical behavior in the normal activities of the snakes, including feeding, was detected during the period after recovery. Forty days after surgery, one specimen escaped unexpectedly, being recaptured 28 days after its escape in a perfect state of health. **Discussion:** Visual function tests suggested that the basic intra-ocular structures remained functional. Additionally, the escape and subsequent recapture of the fugitive animal supplies additional evidence for the maintenance of vision demonstrating the technical efficiency of the procedure. The aspiration of nuclei using the spectacle window technique was considered successful from a technical aspect and is a feasible clinical intervention for cataract treatment in snakes.

Acknowledgements: We thank Prado, LP and Ferrarezzi, H for the critical review of the abstract

5.02 Determination of serum levels of corticosterone and testosterone in males of *Bothrops jararaca* submitted to venom extraction

Grego KF[1], Viau P[2], Guimarães MABV[3], Oliveira CA[2], Catão-Dias JL[3,4]
[1]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil; [2]Departamento de Reprodução, [3]Departamento de Patologia Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brazil; [4]Fundação Parque Zoológico de São Paulo, SP, Brazil

Introduction: Due to the decrease in the number of poisonous snakes received each year by the Instituto Butantan, the animal facility of the Herpetological Laboratory found it necessary to study the maintenance of poisonous snakes in captivity, with the aim of self-sufficiency. The venom obtained at the animal facility is used to produce anti-venom serum and for biomedical research. At the animal facility, there are approximately 100 pit vipers, and 60% are submitted monthly to venom extraction. **Objectives:** The aim of this study was to determine the serum levels of corticosterone and testosterone in male pit vipers in response to the venom extraction routine and delineate more efficient strategies for the maintenance of breeder snakes. **Methods:** In this study, we used 17 males born in captivity that were 3 years old, and they were divided into groups A, B and C. The 7 snakes of group A did not have their daily routine changed, being kept in the same room in which they were born, and were not submitted to venom extraction. The animals of groups B and C were transferred from the room where they were kept since birth to a production room. The snakes of group C were submitted monthly to venom extraction, and the snakes of group B, although not being submitted to venom extraction, witnessed a continuous movement of people inside the production room on extraction days. Every 2 months, for a period of one year, blood was collected by ventral caudal venipuncture at two different moments. The first blood sample was collected 120 seconds after physical restraint to establish the basal serum levels of testosterone and corticosterone, and the second blood sample was collected 90 minutes after restraint and/or milking routine to determine the serum levels of the hormones after these procedures. The serum levels of testosterone and corticosterone were determined by radioimmunoassay at the "Laboratório de Dosagens Hormonais do Departamento de Reprodução da FMVZ/USP." **Results and Discussion:** The results showed that all animals of the 3 groups, after physical restraint and milking routine, exhibited higher levels of corticosterone indicating that these procedures can be considered a stress agent. The higher levels of corticosterone were found in animals of group C, followed by animals of group B. In relation to the serum testosterone levels, we noticed that there was no significant difference in hormone concentration between the first and second blood samples. The end of the study coincided with the beginning of the spermatogenesis period (summer), and we noticed that whereas group A showed a 41% increase in testosterone levels, groups B and C showed a decrease of 67% and 91%, respectively. We therefore suggest that an adrenocortical response to a stress agent during spermatogenesis period diminishes serum testosterone levels, impairing reproduction in snakes kept in production rooms, and that it is very important to maintain male breeders in a an area separate from the production laboratories.

Supported by Fapesp, CNPq

5.03 Studies of *Micrurus* maintained in the Herpetology Laboratory at Butantan Institute: a review of the last twelve years with survival rates (Serpentes, Viperidae)

Antônio AL[1], Fernandes W[2], Zanotti AP[3], Grego KF[2], Sant'Anna SS[2]
[1]Universidade Cruzeiro do Sul, SP, Brazil; [2]Laboratório de Herpetologia, [3]Laboratório de Biologia Celular, Instituto Butantan, SP, Brazil

Introduction: The genus *Micrurus* is known by coral snakes, and two species, *M. corallinus* and *M. frontalis,* are very important to the Instituto Butantan because their venoms are used to produce elapidic anti-serum. However, the poor adaptability of this genus in captivity, associated with small quantity of venom produced by these species, puts the venom reserve in a delicate situation. **Objectives**: The aim of the study was to perform a descriptive analysis of specimens that have been incorporated into the breeding stock of the Laboratório de Herpetologia of the Instituto Butantan (LHIB) to determine if over the last twelve years there has been improvement in the survival rates of these animals in captivity and to examine the factors that can influence survival. **Results**: Between the years of 1996 and 2007, 1075 *M. corallinus* and only 21 *M. frontalis* were received in the animal facility, but this number always oscillated mainly for *M. frontalis* which had no entries in the years of 1996, 2000 and 2002 to 2005. Seventy- seven percent of *M. corallinus* were received at IB during the hotter and rainy season of the year, spring and summer, while 61% of *M. frontalis* were received in the same period above and 30% in autumn. The animal facility received a higher number of females of *M. corallinus* (55%) and only in the spring was the number of males higher. These sexual differences can be related to the size of the animals (females are usually larger than males), as well to the activity of these animals in the different seasons of the year. As to survival in captivity, there was no relation between the size or sex of the coral snakes and the time kept in captivity, but in the last six years the survival rates increased by 15%. **Discussion**: The maintenance of this genus in captivity still needs to be improved. Changes in the captivity environment are producing good results, but feeding is still a problem to be solved. These twelve years of maintenance demonstrated that the care provided by well-qualified professionals and constant attention for these delicate species are having positive effects.

5.04 Ophthalmopathies in captivity-born colubrid offspring: the importance of their anatomopathologic characterization

Casagrande RA[1], Braz HBP[2], Serapicos EO[2], Sá LRM[1]

[1]Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brazil; [2]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: Ophthalmologic alterations are common in captivity-kept snakes and they may result from genetic alteration, inadequate captivity conditions or imperfect nutritional management, and may arise as well from systemic or localized infectious processes. **Objective:** The aim of the study was to describe the ophthalmologic alterationz observed in colubrid young born in captivity. **Methods:** After incubation at an average temperature of 24.3 ± 2.5º C, only one female offspring of *Liophis poecilogyrus* hatched from the oviposition of 16 eggs which were incubated for 111 days, and it showed microphthalmia of the left eye. After 91 days of incubation, 18 (out of 20) *Oxyrhopus guibei* eggs hatched, of which only one male offspring showed, on the hatching day, exophthalmia of the left eye. The specimen of *L. poecilogyrus* did not feed on anything during 77 days of life, and the *O. guibei* young showed a progressive increase in its eye ball during nine days after hatching, after which euthanasia was performed, followed by necropsy of both snakes. **Results:** Macroscopically, one could observe that in *L. poecilogyrus* the left eye ball was 1mm in diameter and the periocular scales were smaller. In addition, there was maxillary dysplasia characterized by the incomplete closing of the left side of the mouth permitting the visualization of the oral mucous membrane which was edematous and reddish. On the other hand, *O. guibei* showed deformity of the left side of the head, exophthalmia, and a 4-mm-diameter eye ball. The eye surface was opaque, there was mucous secretion around it, and the iris was partially visible. The macroscopic analyses of serial cross-sections of the skull showed in *L. poecilogyrus,* unilateral microphthalmia, maxillary bone asymmetry, and acute bacterial stomatitis at the left mucous-cutaneous junction. In *O. guibei's* skull sections one could observe ulcerative keratitis with anterior acute uveitis, hypopyon, degeneration of the structures of the posterior chamber, neuritis, dermatitis, and dacryoadenitis with absorption of the adjacent bones. No anatomopathologic alterations could be observed in the animals' other organs. **Discussion:** This is the first account of microphthalmia and maxofacial dysplasia in *L. poecilogyrus* offspring. Microphthalmia is the most common congenital alteration in snakes; it can be uni- or bilateral and it is often accompanied by a head malformation, with maxofacial dysplasia. Keratitis can be the result of traumatic injuries or of the extension of localized infection in the spectacle and subspectacular spaces of snakes, while uveitis with hypopyon is related to systemic bacterial infectious processes. In the case of *O. guibei,* the ophthalmologic injuries were attributed to an acquired localized bacterial infection, for which predisposing factors were undetermined. The cause of ophthalmopathies in reptiles is often undetermined, but genetic and nutritional factors, exposure to teratogenic agents and temperature conditions during gestation and egg incubation may be related to the appearance of such disorders. Thus, this study highlights the importance of anatomopathologic examination, since it permits the clear characterization and differentiation of congenital and acquired ophthalmopathies, aiding in the reproductive management of snakes in captivity.

5.05 Use of Whitten effect in N: NIH mouse production in the Butantan Institute, Central Animal Facility

Rodrigues UP, Rocha da Silva AP, Mattaraia VGM, Ghiuro EJV, Moreira VB, Távora MFCLF
Divisão Biotério Central, Instituto Butantan, SP, Brazil

Introduction: According to reproduction systems in animal facilities, weaned females remain grouped until mating age. Due to this grouping, females show an irregular estrous cycle, suggesting the presence of a female "pheromone" disturbing the cycles. **Objectives**: In the present work, we used a hormone called "pheromone," secreted by foreskin glands, and released in male urine (Whitten effect) to establish estrus synchronization in mice, obtaining controlled mating and thus expected day of the birth. The practical viability of the method implemented on a large scale was analyzed, with the aim of reducing work and costs. **Methods:** Two hundred twenty-five female N:NIH mice were used, aged up to about 60 days. They were kept in cages with wet straw, with urine of a male mouse, three days before mating. After the third day, females were placed in an intensive mating system (5 female to 1 male), and impregnation was confirmed by the observation of a vaginal plug and fluid. **Results and Discussion**: An analysis was made of the number of pregnant females as well as the number of young that were born on the expected date. The results obtained were significant, since the percentage of estrus-synchronized females was 90.7% of the mated females, confirming estrus synchronization, and the viability of the method used.

6. Education and Science Diffusion

6.01 Attracting and retention power of Biological Museum exhibition

Meireles FA, Schunck AS, Cizauskas I, Iszlaji C, Bizerra AF
Museu Biológico, Instituto Butantan, SP, Brazil

Introduction: The permanent exhibition of the Butantan Institute Biological Museum possesses great attractiveness and recognition among the national and international public. Its last reorganization occurred in 2001, showing 85 exhibits that included dioramas with living animals (biodioramas), dioramas with replicas, informative posters, cases with biological pieces and scale models. Although these exhibits are quantitatively attractive, little is known about patterns of use by the public. **Objectives:** The aim of the study was to identify temporal and spatial patterns of use for the permanent exhibition of the Biological Museum by non-student visitors. **Methods:** In order to observe the patterns of spatial and temporal use of the exhibition and to determine which objects and exhibits exert greater attraction (number of visitors who stop at the exhibits) and retention (amount of time spent by visitors at the exhibits), we recorded the movements, the time spent, and the behavior of 15 visitors. Each selected visitor was followed by a scientist during his/her visit. We recorded in a sketch the route chosen (tracking), the time spent in each one of the 85 exhibits (timing) and the behavior displayed (interaction with animal, friend or guide, reading posted information, etc.). At the end of the visit, the visitor was interviewed. **Results:** Each visitor interacted, on average, with 66% of the exhibits, and spent about 27 minutes per visit. Of the 85 exhibits, 13 were considered highly attractive (they attracted more than 75% of the visitors), such as the dioramas of the exotic species *Python molurus* and *Naja haje*. The women interacted with a higher number of exhibits. Regarding the retention power, the osteology case held back each visitor for a longer time, followed by the dioramas of the anacondas and iguanas. On average, each visitor remained 10.6 seconds in each one of the exhibits visited (77% of the total exhibits). Males and females and different age groups did not show any variations in retention power of the exhibits. The biodioramas of "caiçaca," "escorpião-marrom" and "perereca-babenta" displayed the least holding index. The posters were totally ignored. In regard to behaviors observed, the interaction with the animals was most frequent (100% of the visitors), followed by the interaction with guides (50%) and friends (50%). **Discussion:** The analysis of the data obtained using these methods allows us to both elaborate better materials and evaluate existing materials regarding the public communication of science. It is important to point out that such research, when analyzing the "visitor/museum" interaction allows important reflections for the education and communication staff of the institution, because they point out elements of evaluation and reorganization for an effective exhibition. Getting to know the audience thoroughly and to understand how it interprets the exhibition is essential for obtaining a better quality work from the education staff of museums.

Supported by Fundação Butantan

6.02 The Biological Museum exhibition audience

Schunck AS, Meireles FA, Cizauskas I, Iszlaji C, Bizerra AF
Museu Biológico, Instituto Butantan, SP, Brazil

Introduction: The permanent exhibition of the Butantan Institute Biological Museum receives annually about 200 thousand visitors, being considered one of the most attractive tourist places of São Paulo City. The public of this exhibition is diversified, comprising students and open groups, among others. Although this public is numerically known, data about its composition are lacking. **Objectives:** To define the visiting public profile of the Butantan Institute Biological Museum, as regards age, sex and group formation. **Methods:** Data about estimated age, sex and formation of each visiting group were recorded in a spreadsheet. The visitor age was included in one of the five proposed categories: child (0-12 years old), adolescent (13-20), adult (21-40), middle-aged adult (41-60) and senior adult (more than 60). The data were collected every day of the week, for 15 days, during the attendance schedules. The period of school recess was prioritized. **Results:** A total of 3994 individuals were observed (approximately 16,000 monthly visitors) and females and males had similar representations. The age categories with higher number of visitors were "children" (0-12 years old) and "adults" (21-40). The visiting groups were mainly composed of threesomes (n=313, 939 visitors) and foursomes (n=231, 924 visitors). Only 34 people visited the museum individually. **Discussion:** In the present research, men and women did not show differences in the visitation frequency, helping to put in check the idea demonstrated by research on the public's perception of science, that women have less interest in scientific subjects. Children up to 12 years old and adults of 21-40 years old comprised the majority in this period, and generally, they toured the museum in threesomes. These data are important for the museum staff, as the interactive exhibits do not offer a collective performance. As the education team faces the process of learning (considered here in its cognitive, affective and conative dimensions) as inserted in the social context of the visitor, it is necessary to change the present exhibition. It would be interesting if the museum staff offered other possibilities of interaction with the exhibits, which could be developed in a group.

Supported by FINEP/MCT

6.03 Scientific dissemination by the Microbiology Museum of the Instituto Butantan outside of its physical space

Henrique BC[1], Casadei K[1], Pereira FF[1], Imparato BA[1], Vieira JLA[1], Bizerra AF[2], Inglez GC[1], Tino de Franco M[1]

[1]Museu de Microbiologia, [2]Museu Biológico, Instituto Butantan, SP, Brazil

Introduction: Scientific information reaches people through different media, such as TV, radio, newspapers, internet and scientific publications. At the same time, museums and science centers are places that intend to show how scientific knowledge can be constructed in an interesting way. In this context, the Microbiology Museum of the Butantan Institute comes to join those communication vehicles, with the purpose of approaching people about scientific knowledge by means of an informal, playful and pleasant dialogue. **Objective:** The objective was to promote scientific information and, through this action, make possible social inclusion. Microbiology Museum conducted some practical scientific activities, outside of its physical space, directed at the public in general. These activities took place in Health and Citizenship Fairs, promoted by several entities in different cities of São Paulo State, and in the park grounds of the Butantan Institute during events such as the National Week of Science and Technology. **Methods**: One of these activities, called "Modeling DNA", aimed at the construction of a DNA molecule using simple materials such candies (jelly beans), wire and toothpicks. The objective was to demystify the complexity of a DNA molecule and to elucidate its importance for life. Another activity is an exposition called "Hygiene and a Healthful Life," where people can visualize living microorganisms in a drop of untreated water and bacteria in a drop of yogurt, learning that microorganisms exist, even when not visible to the naked eye. In this exposition, we taught that microbes can live in many places and environments, such as wells of untreated water, water storage vessels that are not cleaned, in poorly washed foods and also on our hands, causing illnesses. However, we also taught that some microorganisms can be extremely beneficial and essential, for example, in some foods (such as yogurt), without causing any harm to human beings. Finally, we also organized a "Fishing Pond," where people fished for pots containing very small images of microbes from a simulated lake, that later are visualized under a microscope. In this event, we intended to emphasize the understanding that microorganisms can contaminate dams and lakes, representing a danger for anyone who drinks untreated water from these places. **Results and Discussion:** The itinerant activities of the Microbiology Museum are enclosed in a project of scientific dissemination developed by its team of professionals. The intention is to foster better conditions for people to exercise their citizenship, enabling them to request authorities for public policies in basic sanitation, seeking the suppression of many illnesses that could be prevented. We believe that our efforts can contribute to the spread of scientific knowledge and to improve people's involvement in government decision-making concerning social matters.

Supported by FINEP, FAPESP, Fundação Butantan

6.04 Museum of Microbiology exhibition "The Great Epidemics": public preliminary research

Imparato BA[1], Casadei K[1], Henrique BC[1], Vieira JLA[1], Pereira FF[1], Inglez GC[1], Tino de Franco M[1], Bizerra AF[2]
[1]Museu de Microbiologia, [2]Museu Biológico, Instituto Butantan, SP, Brazil

Introduction: Great epidemics occurred in the past as a result of poor urban sanitary conditions and the lack of scientific knowledge about disease etiology. Despite the great science and technology improvements, it seems that our world is not free from the ancient threat of epidemics. Science information is crucial to help people to act accordingly in a potential health threat situation. The Museum of Microbiology from Butantan Institute, according to its educational goal, has planned an itinerant exposition telling about feared diseases that caused epidemics such as plague, smallpox, Spanish flu, meningitis and AIDS. **Objective:** Our aim was to obtain more information about public expectations for an epidemics itinerant exposition (conceptual evaluation) in terms of public preferences, interests, attitudes, concepts and knowledge in order to better develop this exposition. **Methods**: Sixteen interviews were performed with the public after Museum visitation. We used a questionnaire with five open questions collecting data about the public's age, sex, schooling, knowledge about epidemics and expectation for a possible exposition in this field. **Results:** Fifty-six percent of the people analyzed were women, fifty percent had only a 3rd grade education, and they were between 28 and 50 years old. Most of the people interviewed did not know the existence in the past of the epidemics described above and could not distinguish between pandemics and epidemics. The transmission, prevention and the historical aspect of the epidemics were cited as the most attractive subjects to be explored. **Discussion:** This work has brought some important information about the public expectation for an exposition on the great epidemics. In spite of the small number of interviews, we could observe some people needs and desires. We will extend the number of interviews in order to develop an exposition that corresponds to the public's expectations. We intend to avoid in the future the same catastrophic effects caused by epidemics in the past through scientific information.

Supported by FINEP, FAPESP, Fundação Butantan

6.05 First "Vital Brazil Week" of the Instituto Butantan Historical Museum – historical trajectory and scientific memory

Fernandes SCG[1,2], Solosando A[1,2], Freitas AC[3], Iszlaji Jr A[1], Massagardi LF[1], Vital RP[1], Godoy AC[1], Santos AE[1]

[1]Museu Histórico, [2]Laboratório Especial de História da Ciência, Instituto Butantan, SP, Brazil

Intoduction: In April, 2007, Vital Brazil completed 142 years of existence and, among the various ways of observing the event, Institute Butantan celebrated the date discussing its importance in the scientific scene, in a partnership with Duna Dueto Publishing House, with the release of the children's book *Vital Brazil*, by Nereide S. Santa Rosa. This work was made possible with the help of many professionals of the Institute Butantan and the Historical Museum. **Objectives**: Together with the research being carried out with the public regarding the main characters in Brazilian science, the aim of this work was to discuss the importance of the scientist's research in his day and nowadays. **Methods**: The Museum's team and the publisher's staff developed together activities that would be incorporated into visits by the public, including video presentations about Vital Brazil and Ofidism and discussion on themes related to national science and the scientific contribution of Vital Brazil, in addition to recreational activities. Supported by the monitorship, which allowed us to make some considerations regarding the lack of knowledge of most of the public about the origin and the development of the research in the social, ecological, economic and medical dimensions, the Historical Museum chose, initially, to work with students of the Fundamental School. Some groups already scheduled to visit the Institute were contacted and invited to take part, having as selection criteria the time available to visit and the number of students. About ten schools participated during the last week of April, but the activities were extended also into the first week of May, when we served all those who showed interest in our plan: teachers, monitors, families and students. The video mixed recent and old images and scenes of the Belgian movie "Death that Spies" from the 1950s, relating uses of the spaces of the Institute (Central Serpentary, Department of Serum, Research Laboratories, etc.) and discussing the stories of the social subjects and the techniques and technologies employed. **Results**: The release of the book *Vital Brazil* and the exposition that presented the impressions of the students, through drawings, ended the celebrations. As a result, we verified that the video was accepted and requested to be used as a didactic resource in the classroom, by the teachers, as the discussions demand a great preparation from the part of those who conduct them, making the monitors a central figure of the activity. **Discussion**: To monitors and employees, the practice to expand the monitorship, in addition to the opportunity to show the public part of the historical collection never before explored, was decisive in creating thematic monitorships in the Historical Museum. Nowadays, some of the monitors are working to report on the 1st Vital Brazil week, which will probably be part of the Museum's website, offering thanks to the participants and describing the activities of the Historical Museum.

Supported by Divisão de Desenvolvimento Cultural, Fundação Butantan, Duna Dueto Editora
Acknowledgement: Laboratório Especial de História da Ciência

6.06 Old suppliers and the reception of snakes- Instituto Butantan

Calleffo MEV[1], Fernandes SCG[2, 3]
[1]Laboratório de Herpetologia, [2]Museu Histórico, [3]Laboratório Especial de História da Ciência, Instituto Butantan, SP, Brazil

Introduction: Since the beginning of XXth century, the Butantan Institute has received serpents coming from various places in Brazil. This work was initiated in the times of Vital Brazil with the exchange of serum for snakes. Since the 1960s, the exchange no longer exists, and the service to the suppliers has been taken over by the Reception of Snakes of the Laboratory of Herpetology, old Section of Records. The Reception is where animals brought to the Institute are received, which contributes to the Collection of Snakes, and arranges experiments of other laboratories and other institutions that work with snakes. The service of the reception is known traditionally by the suppliers of all Brazil. Its function is to record the origin and the data for each animal received and the supplier. **Methods:** In the records of the suppliers, we find information on the specimens donated to the reception of snakes for the past century. The data in the records book, the photographic and written information, notes on remarks of the employees and documents were used to identify the relation between suppliers and the institute and how much to the condition of the release of the serpents and its frequency. **Objective:** The relation between the history of the suppliers and its current motivations are reproduced in these documents that are being computerized. The aim of the work was to establish the relation between locality, supplier and species throughout the history of the institute. **Results:** The animals sent or brought by the suppliers arrive in different conditions and in diverse ways. Until the 1970s, the boxes came by train without cost, and currently, centers of selection, Zoonoses and Armed Forces, among others, make this a courtesy. When the specimens arrive, after the identification, they are directed to other laboratories. In the 1920s, the reception received about 7,200 specimens, 25,000 in the 1950s, 70,000 in the 1980s, and currently we have more than 100 to a thousand suppliers. We already have registered 18,000 suppliers. The archive is available for research by means of authorization. The Reception still provides visits for suppliers, with a tour of the Institute, in the capital and other locations: Juquitiba, Santana of the Parnaíba, Araçariguama and Ibiúna. This service of demonstrating the animals also serves to inform and to guide the community. The specialized employee goes with them, with box, “Lutz lassos,” hook for collections and didactic material. If it is to be the first encounter, instructions on collections and prevention of accidents are passed out. The Butantan does not recommend campaigns or indiscriminate collections of animals in the nature, and also does not supply material for collections, but it offers support for the current suppliers. Without the exchange, the serum donation is controlled and directed, only if necessary, for some suppliers and places. **Discussion:** This work of the communities still contributes to the institute, in a traditional and friendly service, and the data allows them to know the animals, the geographic distribution, accidents, old and new suppliers, new areas that need a greater attention, beyond being the basis of reports of the entrance and the destination of the animals that are submitted to the IBAMA every month.

6.07 The Rural School, the recovery of the collection and the memory in the Instituto Butantan

Iszlaji AJ[1], Solosando A[1,2], Fernandes SCG[1,2]
[1]Museu Histórico, [2]Laboratório Especial de História da Ciência, Instituto Butantan, SP, Brazil

Introduction: The Rural School of the Butantan Institute, was created with the objective of attending to internal functions and the external community. During the years 1932 to 1943, a teacher and director, Noêmia Saraiva Matos Cruz, recorded photographs of classes, field activities, student contests and other Rural Schools of Brazil's countryside. These pictures were attached to the technical collection of the Historical Museum in 2004, and since then were not researched. We know that this is part of the trajectory of the school that nowadays is the State School Alberto Torres, located at Butantan's entrance. **Objectives:** Our objective was to identity and organize the collection of photographs, in view of two central aspects of this research: 1. Importance to the rural study in São Paulo using as reference the activities developed at the Rural School of Butantan and 2. Possible ways for the preservation and conservation of materials necessary to make them available for research. **Methods:** We began by digitizing the pictures that were attached to albums and in miscellaneous forms, which will support research but prevent contact with the original photographs. In view of the lack of resources and proper materials, storage was contemplated with the objective of developing alternative solutions with regard to the condition of available space, protecting the pictures more regularly from environmental effects such as dust, light and climatic changes. **Results and Discussion:** At the moment, we digitized four full albums, containing around four hundred pictures of the Rural School of the Butantan Institute and Rural Schools of Minas Gerais and of the countryside in São Paulo, and we carried out the first recordings with the objective of identifying solutions for the accessibility these photographic records.

Supported by Fundação Butantan

6.08 Snakebites and casualties in the 1990s in Brazil: focus on the variable "textbooks"

Lucas M[1], Bizzo N[2], Monteiro P[3]
[1]Museu Biológico, Instituto Butantan, SP, Brazil; [2]Faculdade de Educação, Universidade de São Paulo, SP, Brazil; [3]Instituto de Saúde, SP, Brazil

Introduction: Methods and procedures for field management of venomous snakebites before arrival at the medical facility are part of the science curricula for basic education in Brazil. Many science textbooks have provided misleading information, such as wounding the victim with multiple incisions and subsequent suction of the blood, and the use of a tourniquet. The same science textbook that brought information about the danger of direct contact with blood, for the high risk of disease transmission (AIDS, hepatitis, etc.), brought information about the "safety" of "drinking" somebody else's blood with snake poison. The origins of these recommendations were from a report prepared by the *American National Research Council* of the *National Academy of Sciences* for the *American Red Cross* in1979, based on 1927-8 experiments carried out in San Antonio, Texas with rattlesnake (genus *Crotallus*) snakebites. Brazilian recommendations seem to derive from these. Now it is well established that these practices are not recommended, and it is very likely that the effects are different from those expected. Progressive swelling can turn a constriction band into a tight tourniquet, with severe consequences, as amputations and pulmonary thromboembolism. In 1995 Brazilian publishers were invited to submit textbooks for an official assessment carried out by the Ministry of Education, as a qualifying step prior to sales to public schools,(including rural areas). The official results were published in1996, with a major public reaction, which included some nationwide TV news. After that, such errors were banned from science textbooks**. Objectives:** Since this period, experts recommend to move the victim as soon as possible to a medical facility, to rest the affected extremity, to decrease physical exertion in any possible way, and by no means hurt the victim with incisions or tourniquet**. Methods:** The number of casualties from snakebites declined in the subsequent years in Brazil, like the official health statistics revealed (DATASUS-SIH). **Results:** It is not easy to know the real number of casualties caused by snakebites in Brazil. In the period 1990-3, there were 81,611 snakebites officially recorded, but the real number may be much higher, as compulsory notification is frequent only when the victim seeks medical assistance. In the period 1986-94, around 110 deaths occurred per year in the country. In the years 1993-5, an average of 75 of these deaths occurred inside hospital facilities. The same database shows that in the years 1996-9, there were around 45 deaths per year. In other words, there was a significant reduction in the mortality (almost 50%), which is consistent with the hypothesis of change in field management procedures. Especially in the cases of *Bothrops* snakebites, which are almost 92% of the total in Brazil, theses changes could explain a lower death rate. **Discussion:** Although it is important to carry out surveys to test other hypotheses, which could possibly explain such reduction, we believe that this improvement was the result of changes in field management of snakebites as a consequence of the massive change of science textbooks all over the country. Science education can play a major role in improving public health and reducing mortality.

7. Cellular Biology

7.01 Glutamatergic signaling in the rat pineal gland

Villela DC[1], Moutinho AA[1], Abrahão MV[1], Amaral FG[3], Lima L[2], Cipolla-Neto J[3], Afeche SC[1]

[1]Laboratório de Farmacologia, Instituto Butantan, SP, Brazil; [2]Departamento de Farmacologia, [3]Departamento de Fisiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil

Introduction: The mammalian pineal gland synthesizes melatonin only in the dark in response to a noradrenergic stimulus. Several neurotransmitters modulate melatonin synthesis, among them glutamate, which is known to act as a negative regulator, because it inhibits N-acetyltransferase, a key enzyme in melatonin synthesis. **Objectives**: The aim of this work was to characterize the receptors and the transduction pathways involved in the inhibition of glutamate-induced melatonin synthesis. More specifically, we investigated the participation of the nuclear factor NFκB, a known transcription factor activated by glutamate in the central nervous system. **Methods**: Pineal glands were isolated from Wistar rats and maintained in culture for 48h, in BGJb medium. They were then stimulated by 1μM norepinephrine plus glutamate at various concentrations or by the glutamate receptor agonists AMPA, S-3,5 DHPG (class I metabotropic agonist), and L-CCG (class II metabotropic agonist). The NMDA receptor antagonist MK-801 was also used in combination with glutamate. Melatonin synthesis was evaluated by HPLC with electrochemical detection. NFκB was analyzed by the eletrophoretic mobility gel shift assay. Glutamate receptors were characterized by RT-PCR. **Results:** Glutamate reduced melatonin synthesis at every concentration tested. MK-801 did not show any effect on the inhibitory effect of glutamate; in contrast, AMPA and L-CCG significantly reduced melatonin synthesis. mRNA for the receptors AMPA, mGluR1, mGluR2 and mGluR3 were found in the pineal gland. Glutamate activated the nuclear factor NF-kB. **Discussion:** Glutamate reduces melatonin synthesis, and this response seems to be mediated by AMPA and class II (mGluR2 and mGluR3) metabotropic receptors. Glutamate activates the nuclear factor NF-kB in pineal gland, but its role in melatonin synthesis remains unclear.

Supported by FAPESP, CNPq

7.02 Noradrenaline activates venom gland of the snake *Bothrops jararaca* by regulating transcription factors activation

Luna MSA[1], Ferreira ZS[2], Yamanouye N[1]
[1]Laboratório de Farmacologia, Instituto Butantan, SP, Brazil; [2]Departamento de Fisiologia, Instituto de Biociências, Universidade de São Paulo, SP, Brazil

Introduction: Viperidae snake venom gland has a central lumen where all venom produced is stored. Secretory cells are stimulated for a new cycle of venom synthesis after emptying the lumen either by manual extraction or biting. Concurrent with an increase in the synthesis of mRNA and protein, there is an increase in the size of secretory cells, where flattened quiescent cells become columnar (peaks at 4 days after milking). The venom production cycle is long and lasts around 30-50 days. Activation of noradrenergic innervation is essential for triggering the venom production cycle in the *Bothrops jararaca* venom gland by stimulating both α- and β-adrenoceptors [1, 2]. We showed that stimulation of both α- and β-adrenoceptors affects the synthesis of proteins in the venom gland, which are probably involved in activating the venom gland rather than the synthesis of toxins. Besides, we showed for the first time the presence of activated transcription factors NFκB and AP-1 in quiescent stage venom gland. Venom extraction is able to increase the activation of NFκB and AP-1 in secretory cells of venom gland. **Objective:** The aim of this study was to determine whether noradrenaline by stimulating both α- and β-adrenoceptors is able to activate both NFκB and AP-1 transcription factors. **Methods:** Nuclear extracts were prepared from disaggregated secretory cells obtained from the venom glands of female snakes whose venom was not extracted previously (quiescent stage). A total of 2 to 3 x 10^6 cells were incubated in Krebs-HEPES solution containing noradrenaline (10^{-4}M), phenylephrine (3.10^{-4}M) or isoprenaline (3.10^{-4}M) for 30 min at 30°C. The activation of transcription factors was analyzed by the electrophoretic mobility shift assay. **Results:** Noradrenaline was able to increase the activation of both NFκB (56.15, *N*=2) and AP-1 (15.37, *N*=2) in quiescent secretory cells of snake venom gland. Phenylephrine and isoprenaline, respective agonists of α- or β-adrenoceptors, were able to increase the activation of NFκB (17.82 ± 3.97, *N*=6; 20.20 ± 6.79, *N*=4, respectively). However, only isoprenaline was able to increase the activation of AP-1 (23.42 ± 7.78, *N*=3). In fact, phenylephrine decreased the activation of AP-1 (-16.67 ± 2.58, *N*=7). Competition studies using unlabeled NFκB or AP-1 double-strand oligonucleotides showed the specificity of NFκB/DNA and AP-1/DNA binding interaction, respectively. **Discussion:** Our results suggest that the activation of noradrenergic innervation by venom extraction releases noradrenaline that modulates the activation of transcription factors by stimulating both α- and β-adrenoceptors and consequently regulates the synthesis of proteins of the venom gland, which has a crucial role in modulating venom gland activation.

References: 1. Yamanouye *et al.* Life Sci., 67: 217, 2000; 2. Kerchove *et al.* J. Exp. Biol., 207:411, 2004.

Supported by FAPESP, Fundação Butantan

7.03 Immortalization of venom-producing cells of the Viperidae snake *Bothrops jararaca*: standardization of ideal conditions

Hortencio TMA[1], Carneiro SM[2], Yamanouye N[1]
[1]Laboratório de Farmacologia, [2]Laboratório de Biologia Celular, Instituto Butantan, SP, Brazil

Introduction: Long-term primary culture of secretory cells of the venom gland has been well established. These cells are viable for 21 days and are able to produce and secrete venom into the medium with biological activity similar to that of the venom obtained from the snake. **Objective:** The aim of this study was to develop methods for immortalization of secretory cells of the venom gland of *Bothrops jararaca*, in order to obtain a functional cell line that can produce and secrete venom into the medium. **Methods:** Both adult and newborn *B. jararaca* were used in this study. Secretory cells of the venom gland were obtained as described elsewhere [(1)]. The cells were plated in a 24-well plate ($2x10^6$ cells/well) in 1mL of DMEM containing 40mM sodium bicarbonate, supplemented with 10% FBS and antibiotics (100U/mL of penicillin and 100µg/mL of streptomycin). The cells were maintained at 30ºC in a humidified incubator (5% CO_2). Erythrocytes present in the dispersed cell preparation were excluded by Ficoll density gradient centrifugation. After 24h, cells were transfected with either the plasmid pSVori$^-$ which contains the oncogene for the expression of the viral protein T-large, or with the plasmid pCI-neo-hTERT which contains the gene for the expression of human telomerase reverse transcriptase, or co-transfected with both plasmids. **Results:** Cultures of secretory cells from adult snakes survived for 34.8 ± 3.3 (n = 6) days when transfected with pSVori$^-$, 32.7 ± 1.0 days (n = 3) when transfected with pCI-neo-hTERT and 33.0 ± 1.0 days (n = 3) when co-transfected with both plasmids. However, when erythrocytes were removed, cultures of secretory cells transfected with pSVori$^-$ or co-transfected with both plasmids were maintained up to now (more than 100 days). These cells are non-adherent cells and sometimes can form acini. Morphological analysis of acini revealed the presence of collagen-secreting cells. In this culture, the adherent acini apparently were bound to the substrate through these collagen- secreting adherent cells. Secretory cells from newborn snakes transfected with pSVori$^-$ or co-transfected with both plasmids were maintained for 140 days (n = 1). After 30 days of culture, these cells start to form acini. For cell cultures from adults and newborns, the number of cells decreases up to the 6^{th} day and then increases up to the initial concentration. **Discussion:** Co-transfection with pSVori$^-$ and pCI-neo-hTERT did not differ from transfections with the plasmid pSVori$^-$ or pCI-neo-hTERT alone. Nevertheless, cell proliferation rates in all cases were very low. These results were obtained without the addition of co-factors such as those used for the long-term primary cultures established before. The presence of collagen-secreting adherent cells indicates that the use of these cells as feeder cells may be desirable for improving cell culture conditions. In addition, secretory cells from newborns seem to be more appropriate for establishing an immortalized cell line.

Reference: 1. Yamanouye *et al.* Nature Protocols, 1: 2763, 2007.
Supported by CNPq, FAPESP, Fundação Butantan

7.04 Endocytosis-dependent cytotoxic effect of crotamine

Kerkis I[1], Hayashi MAF [2,3], Nascimento FD[4], Kerkis A[5], Oliveira V[6], Oliveira EB[7], Pereira A[1], Rádis-Baptista G[8], Nader HB[9], Yamane T[10], Tersariol ILS[11]
[1]Laboratório de Genética, [3]Centro de Toxinologia Aplicada – CAT/CEPID, Instituto Butantan, SP, Brazil; [2]Departamento de Farmacologia, [6]Departamento de Biofísica, [9]Departamento de Bioquímica, Universidade Federal de São Paulo, SP, Brazil; [4]Med Discovery S.A., Geneva, Switzerland; [5]Clínica e Centro de Pesquisa em Reprodução Humana "Roger Abdelmassih", SP, Brazil; [7]Departamento de Bioquímica e Imunologia, Universidade de São Paulo - Ribeirão Preto, SP, Brazil; [8]Centro de Ciências Biológicas, Universidade Federal de Pernambuco, PE, Brazil; [10] Instituto de Pesquisas Energéticas e Nucleares, CNEN, SP, Brazil; [11]Centro Interdisciplinar de Investigação Bioquímica, Universidade de Mogi das Cruzes, SP, Brazil

Introduction: Crotamine is one of main toxic components of *Crotalus durissus terrificus* venom which belongs to a group of closely related small, nonenzymatic, basic polypeptides which cause necrosis of muscle cells. Recently, we have shown that crotamine is internalized by endocytosis by binding to heparan sulfate proteoglycans on the cell membrane [(1)]. **Objectives:** In order to investigate the mechanism of in vivo cytotoxic effects of crotamine, *in vitro* studies using its high concentration were performed. **Methods:** Fluorescent Cy3 conjugated crotamine was prepared using the Fluorolink™ Cy3-reactive dye. In vivo tissue and cell localization of crotamine was assessed with a confocal laser scanning microscope in order to observe the lysosomotropic properties of crotamine, we used the acridine orange (AO) uptake and relocation methods, described previously. For chromosome studies, the protocol was the same as that described previously (Kerkis et al., 2004). **Results and Discussion:** In the present work, we demonstrated that crotamine is distributed to several tissues after intraperitoneal injection into mice, such as lung, liver, spleen and muscles. Our results show that crotamine accumulates in lysosomes of CHO-K1 cells by a concentration- and time-dependent process. The heavy accumulation of crotamine inside lysosomes triggers lysosomal-membrane permeabilization with release of both lysosomal enzymes and crotamine into cytosol. Also, it was observed that the released crotamine penetrates into the nucleus, where it binds with chromatin. DNA interaction with histone can be dislodged by crotamine binding and, consequently, promotes a chromatin decondensation state that in turn can enhance the sensitivity of DNA to nucleases inducing cell cycle arrest. Eventually, both lysosomal-membrane permeabilization process and nuclear binding of crotamine can contribute to inducing cell death. For an accurate understanding of the molecular mechanism(s) involved in the cell death pathway induced by crotamine, a further detailed investigation will be required.

References: 1-Nascimento *et al.*, JBC, 2007; Kerkis *et al.*, FASEB J, 18: 1407-09, 2004.
Acknowledgement: We thank the Center for Applied Toxinology (CAT/CEPID) for the use of the mass spectrometry facility and also Dr. Katsuhiro Konno for the technical expertise.
Supported by FAPESP

7.05 Detection of estrogen receptor beta (ERβ) and matrix metalloproteinases MMP-2 and MMP-9 in the dorsal skin of the anuran amphibian *Pipa carvalhoi* during egg implantation

Fernandes TL[1], Antoniazzi MM[1], Sasso-Cerri E[2], Egami MI[3], Boletini-Santos D[4], Lima C[4], Miraglia SM[3], Jared C[1]

[1]Laboratório de Biologia Celular, [4]Laboratório Especial de Toxinologia Aplicada-CAT/CEPID, Instituto Butantan, SP, Brazil; [2]Faculdade de Odontologia, Universidade Estadual Paulista, SP, Brazil; [3]Departamento de Morfologia, Universidade Federal de São Paulo, SP, Brazil

Introduction: Amphibian integument is involved in many different functions such as respiration, ion and water transport, sensorial reception, chemical protection and reproduction. Among anurans, most species lay eggs directly into water or moist places, and parental care can be present or not. Other species, however, developed different forms of mobile parental care in which the eggs are carried by one of the parents, allowing it to be free to move around and, at the same time, giving protection to the clutch. *Pipa carvalhoi* is an aquatic anuran from the family Pipidae. During the reproductive period, the species exhibits a complex courtship ritual, in which the amplexed pair deposits the eggs on the female's back, aided by the male. Within 24 hours, the eggs submerge inside the female's dorsal skin, housing inside chambers, where they develop to tadpoles in about 21 days, in a process resembling trophoblast nidation in mammals' endometrium. **Objective**: In this study, we looked for the possible participation of estrogen receptor beta (ERβ) and of metalloproteinases MMP-2 and MMP-9 in the process of egg implantation in *P. carvalhoi*. **Methods**: Fragments of the dorsal skin of females without eggs, immediately after posture and in the first day after posture were fixed in Bouin's, dehydrated and embedded in paraffin. Sections 14 μm thick were stained with HE, and N.M.C. trichrome or submitted to Alcian blue staining at pH 2.5. Other sections were used for immunohistochemistry to detect ERβ, MMP-2 and MMP-9. **Results**: The eggs penetrate into the skin through an invagination of the epidermis, which is changed into a flat-cell monolayer lining the interior of chambers, one for each egg. In order to make space for the chambers, the dermis undergoes a dramatic rearrangement, in which the collagen fibers around the egg chambers are in part substituted by an amorphous fundamental substance, rich in hyaluronic acid, evidenced by Alcian blue after incubation with hyaluronidase. The three proteins tested by immunohistochemistry were detected in the skin. ERβ was present mainly inside the cutaneous glands and blood vessels, but was also detected in the epidermis. Both MMPs were detected spread over the dermis and, more discretely, inside the glands. **Discussion**: The rapid process of egg implantation in the skin, within a few hours, strongly suggests the mediation of biochemical signaling. The fundamental amorphous substance present in the dermis confers more malleability to the skin in order to better accommodate the eggs. The detection of ERβ in the skin indicates the participation of estrogens in the process. The detection of the MMPs indicates that these enzymes are possibly related to the dissociation of fibers of the dermal connective tissue, and maybe also of the epidermal cell junctions. In addition, these enzymes can be associated with the increase in skin vascularization during embryonic development, forming an extensive capillary net around the egg chambers, which was clearly observed from the 14th day until tadpole eclosion.

Supported by CNPq, Fundação Butantan

7.06 Morphological changes and decrease in migration and invasion of breast cancer cells treated with a marine sponge peptide

Rangel M[1], Freitas VM[2], Bisson LF[2], Jaeger RG[2], Machado-Santelli GM[2]
[1]Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil; [2]Departamento de Biologia Celular e Desenvolvimento, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil

Introduction: Cell lines grown in three-dimensional (3D) culture conditions recapitulate essential features of the tissue *in vivo*. Tissues manipulated in 3D conditions are considered tractable cell-based models that allow investigations of possible new drugs and therapies. **Objectives**: We are currently investigating the effects of the depsipeptide geodiamolide H, isolated from the Brazilian sponge *Geodia corticostylifera*, on cancer cell lines grown in a 3D environment. As shown previously, geodiamolide H disrupts actin cytoskeleton in both sea urchin eggs and breast cancer cell monolayers (1). **Materials and Methods**: We used a normal mammary epithelial cell line MCF10A that in 3D assay results in the formation of polarized acini-like spheroids. We also used cell lines derived from breast tumors with different degrees of differentiation and malignancy: MCF-7 positive for estrogen receptor and the pleomorphic Hs578T, negative for hormone receptors. Cells were grown embedded into Matrigel. Spheroids obtained from these cultures were treated on the 10th day with geodiamolide H (20, 120 and 360 nM) for 48h. Control and treated samples were analyzed by light microscopy (H&E), and by laser scanning confocal microscopy of whole-mount preparations stained with rhodamine-phalloidin. **Results and Discussion**: The geodiamolide H affected only the poorly differentiated and aggressive Hs578T cells grown in Matrigel. The peptide-induced phenotypic modifications in this cell line were mostly characterized by disruption of the actin cytoskeleton. This result prompted us to investigate the effect of the geodiamolide H on the migration and invasion of MCF10A and Hs578T cells through time-lapse video microscopy and invasion assays. Time-lapse microscopy showed that the peptide inhibited the migration of Hs578T in a dose-dependent manner, and invasion assays using modified Boyden chambers revealed that geodiamolide H induced a 30% decrease in the invasive behavior of Hs578T cells. MCF10A migration and invasion patterns were not altered by treatment. Our results indicate that geodiamolide H preferentially affected a more aggressive breast cancer cell line in 3D conditions which simulate *in vivo* tissue. Furthermore, the peptide reduced Hs578t cell migration and invasion features, characteristics related to metastasis processes *in vivo*. The fact that normal cell lines were not significantly influenced by treatment with geodiamolide H warrants further studies aimed at its medicinal use.

Reference: 1. Rangel *et al.*, Peptides, 27: 2047, 2006.
Supported by FAPESP, CNPq

7.07 Action of amblyomin-X in cultured cancer cells

Ventura JS, Lapetina DL, De-Sá-Júnior PL, Batista IFC, Ramos OHP, Chudzinski-Tavassi AM
Laboratório de Bioquímica e Biofísica, Instituto Butantan, SP, Brazil

Introduction: Several studies have demonstrated that the saliva of hematophagous organisms contain substances that interfere in the blood coagulation of their hosts. Focusing on this system, a recombinant Factor Xa inhibitor (called Amblyomin-X) was cloned and expressed. Besides its anticoagulant effect, the recombinant protein shows cytotoxicity in several tumor cell lines. **Objectives:** The aim of this study was to treat tumor cells with the inhibitor and to see if treatment interferes with phases of the cell cycle. **Methods:** B16F10, SKmel-28 and MIA-Paca-2 cultured tumor cells were treated with different concentrations of Amblyomin-X. Cytological alterations were analyzed by inverse microscopy, cell viability by flow cytometry and MTT methods, and cell cycle phase by flow cytometry. **Results:** The data obtained showed that Amblyomin-X causes cytotoxicity in a dose-dependent manner in B16F10 and Skmel-28 (melanoma cells). On other hand, the MIA-Paca-2 cell line was more resistant. The flow cytometry results demonstrated a significant increase in G2/M in all three tumor cell lines. **Discussion:** We therefore believe that it is possible that Amblyomin-X causes the disruption of a positive feedback elicited by cancer procoagulant proteins on cancer cells themselves.

Supported by FAPESP, FINEP

7.08 Isolation, characterization and differentiation of equine mesenchymal stem cells derived from adipose tissue

Mambelli LI[1], Santos EJC[2], Borja MC[3], Frazão PJR[3], Maranduba CMC[1], Kerkis A[2], Zoppa ALV[3], Kerkis I[1]

[1]Laboratório de Genética, Instituto Butantan, SP, Brazil; [2]Genética Aplicada Ltda., Centro Inovador de Empresas Tecnológicas, SP, Brazil; [3]Departamento de Cirurgia da Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brazil

Introduction: In horses, stem cell therapy is a promising tool for the treatment of many injuries which are common consequences of athletic endeavor, resulting in high morbidity and often compromising a return to the same level of activities. Recently, the isolation of equine stem cells from peripheral blood and bone marrow was reported. According to our knowledge, there is no information about the isolation of equine mesenchymal stem cells from adipose tissue. **Objective:** The aim of the study was to isolate and characterize equine mesenchymal stem cells (EMSC) from adipose tissue, and to study their differentiation capacity. **Methods:** The cells were isolated from adipose tissue during routine surgery of adult animals using the pre-plating tissue technique. The cells were cultured according to established conditions that maintain adult stem cells (ASC) in an undifferentiated state, previously described by our group [(1)]. The proliferative potential of the cells was evaluated during 23 passages. These cells were induced to differentiate using protocols previously described and developed by us. The differentiated state of the cells was characterized by histochemistry. **Results and Discussion:** Three different strains were successfully isolated from 5 animals. They showed fibroblast-like mesenchymal stem cell (MSC) morphology and high proliferative potential. They demonstrated a expansion rate characteristic of stem cells, growing continuously during 20 passages, without any signs of quiescence. These cells were capable of differentiating into mesoderm derivates: Von Kossa staining revealed the formation of calcified extracellular matrix in differentiated EMSC cultured in osteogenic medium for 21 days, confirming their osteogenic differentiating potential. Adipogenic differentiation was induced using two protocols: a routine one and another developed by us. We showed that both protocols were efficient, although our protocol required a shorter time. Oil red O staining revealed the accumulation of lipid-filled droplets after 7 days, under conditions that we have established, while previously described protocols required about 21 days. The cells were also able to undergo chondrogenic differentiation after 21 days of "pellet" culture, which was evidenced by histological and immunofluorescence studies demonstrating toluidine blue staining of chondrocytes and collagen matrix. Immunofluorescence analysis was performed using specific antibodies against collagen type II and aggrecan. The morphological studies indicated that EMSC can also undergo myogenic and neuronal differentiation. Our data indicate that these cells are similar to the ones isolated from equine bone marrow. These findings demonstrate that isolation of EMSC from adipose tissue is a promising less invasive (compared to bone marrow) method of obtaining stem cells, which can be applied in different cell therapies in horses.

Reference: 1-Kerkis *et al*., Cell, Tissues, Organs, 184:105, 2006.

Supported by FAPESP, Genética Aplicada Ltda.

7.09 Study of spontaneous cell fusion of immature dental pulp stem cells *in vitro*

Lizier NF[1], Maranduba CMC[1], Fonseca SAS[2], Kerkis I[1]
[1]Laboratório de Genética, Instituto Butantan, SP, Brazil; [2]Laboratório de Células-tronco, Clínica e Centro de Pesquisa em Reprodução Humana Roger Abdelmassih, SP, Brazil

Introduction: Currently, the properties of stem cells (SC) are widely explored in regenerative medicine. Adult stem cells (ASC) can be isolated from many tissues including bone marrow, skin, fat, etc.; however, in some cases their isolation is painful for the patient. In order to provide less invasive and painful sources of SC our group conducted a study to isolate SC from dental pulp of "baby teeth." We showed that these cells express some mesenchymal (SH2, SH3 and SH4) and embryonic (OCT-4, Nanog, SSEA3 and STRO-1) SC markers, and they were called immature dental pulp stem cells (IDPSC). Different mechanisms have been proposed to explain the differentiation potential of SC, among which is cell fusion (CF). **Objectives:** Here, we aimed at studying whether these cells would undergo the fusion process in an undifferentiated state *in vitro*. We designed the experiment to verify whether there would be a fusion between the IDPSC or a fusion between an IDPSC and another SC from the bone marrow (BM) of Balb/C mouse. **Methods:** Different samples of IDPSCs were stained with Cell Tracers Vybrants: CM-DiI (red color) and CFDA SE (green color). Mouse BM cells were stained with CFDA SE. IDPSC stained differently were mixed and co-cultured. After 24 and 48 h, the medium was removed and the cells were fixed in 4% formaldehyde for 1 h. The slides were mounted with antifade and DAPI (DNA binding dye) and analyzed under a confocal microscope. FISH investigation was applied using a specific probe for human chromosome 21(LSI 21, Vysis) in order to provide further fusion evidence. **Results and Discussion:** Confocal microscopy showed the presence of IDPSC with only red, only green and yellow fluorescence, indicating that the yellow one was a result of spontaneous CF. Moreover, CF was also observed between IDPSC and mouse BM cells *in vitro*. The FISH analysis revealed cells with 2, 4 and 6 signals of chromosome 21 in the nuclei, suggesting diploid, tetraploid and hexaploid chromosome complements. Our results estimated that 25% of IDPSC underwent spontaneous CF *in vitro* under confluent culture conditions. The CF between IDPSC and BM cells resulted in the formation of hybrid cytoplasm; however, further investigation is needed to make a conclusion about the fusion of their nuclei. Our data suggest that SC culture conditions should be accurately analyzed avoiding those which could lead to CF, such as confluent culture. Different culture media could also contribute to this process as well as passage number and cryopreservation. Moreover, it is necessary to analyze CF after the induction of differentiation *in vitro*, because some of the polyploid cells could be eliminated during this process. It is noteworthy that CF has also its advantages, as it is an essential process for muscular fiber formation, and neuronal and liver cell differentiation.

Supported by CNPq
Acknowledgments to Alexsander Seixas de Souza and Dr. Toshie Kawano of Parasitology Laboratory of Instituto Butantan for providing support with confocal microscopy

7.10 Analysis of engraft of human immature dental pulp stem cells in mouse organs using different inoculation routes

Maranduba CMC[1], Sant'Anna OA[2], Monteiro BG[1], Mambelli LI[1], Lizier NF[1], Fonseca SAS[3], Cerruti Filho H[4], Kerkis A[3], Kerkis I[1]

[1]Laboratório de Genética, [2]Laboratório de Imunoquímica, Instituto Butantan, SP, Brazil; [3]Laboratório de Células-tronco, Clínica e Centro de Pesquisa em Reprodução Humana Roger Abdelmassih, SP, Brazil; [4]Clínica Cera, SP, Brazil

Introduction: Stem cells (SCs) have been widely explored in medical research and have shown promise in tissue repair and regeneration. Although SCs have been injected into animal models, there is no general agreement about the best way to inoculate the animals: intravenously, subcutaneously or intraperitonially? Nor is it known even in which tissue we would observe the major presence of marked SCs according to inoculation route. Our group has isolated a population of SCs from human dental pulp that can express some mesenchymal (SH2, SH3), embryonic (Oct-4, Nanog) and specific-stage antigens (SSEA-3, SSEA-4) as SC markers. These cells were named immature dental pulp stem cells (IDPSCs). **Objectives:** The present study aimed to provide additional information about the distribution of IDPSCs within the different organs and tissues *in vivo* using an animal model. **Methods:** About $5x10^5$ IDPSCs labeled with fluorescent dye DiI Vybrant (red color) were injected intraperitoneally (ip), intravenously (iv) and subcutaneously (sc) into BALB/c mice, which were killed after 28 days. Frozen sections (5μm) were prepared and graft of the cells within mouse organs such as liver, spleen, heart and bone marrow were analyzed by confocal microscopy. Tissue distribution was assessed by using human anti-IDPSC antibody, which was produced in the genetically selected High responder mouse line (H_{III}) and its efficiency previously tested *in vitro*. Fixed sections were incubated with anti-IDPSC antibody followed by incubation with anti-mouse-FITC conjugated secondary antibody. Analysis was performed with confocal microscopy, followed by flow cytometry in order to quantify IDPSC graft within the mouse tissues. **Results:** Merged confocal images showed superposition between DiI labeled cells and areas immunostained with anti-IDPSC antibody (green) confirming the presence of IDPSCs in the organs and tissues examined. This also indicates the high migrating capacity of the cells regardless of inoculation route. Flow cytometry data suggest that the intravenous route was more efficient than ip or sc. Moreover, it seems that engraftment of IDPSCs observed in liver and spleen was greater than in heart and bone marrow. It is noteworthy that the 15 animals studied showed no signs of tumor formation. **Discussion:** It could be demonstrated that IDPSCs possess enhanced migrating capacity, which is relevant for use in cell therapy. Our data indicate that the inoculation route needs to be considered as an important factor since enhanced graft of IDPSC in the tissues studied was observed after iv inoculation.

Supported by CNPq

7.11 Human adult immature stem cells from dental pulp express embryonic stem (ES) cells (Oct-4, Nanog) markers and contribute to the formation of human/ mouse pre-termed chimeras

Kerkis I[1], Abdelmassih S[2], Fonseca SAS[2], Lavagnolli TNC[1,2], Serafim RC[1,2], Santos EJC[1], Mendes CM[3], Ambrosio C[4], Visintin JA[3], Abdelmassih R[2], Kerkis K[2]

[1]Laboratório de Genética, Instituto Butantan, SP, Brazil; [2]Clínica e Centro de Pesquisa em Reprodução Humana Roger Abdelmassih, SP, Brazil; [3]Departamento de Reprodução Animal, [4]Departamento de Cirurgia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brazil

Introduction: Human embryonic stem (hES) cells derived from inner cell mass (ICM) of mammalian blastocysts are rapidly proliferating, pluripotent cells. Since using hES requires embryo destruction, this source of hES faces many ethical objections, and adult stem cells (ASC) are considered as an alternative. We recently reported the isolation of human immature dental pulp stem cells (IDPSC), which express hES cell markers. **Objectives:** In the present study we questioned whether these cells would be able to contribute to the formation of mouse-human chimeras using mouse blastocyst as a reprogramming vector. **Methods**: IDPSC positive for Oct4 and Nanog, markers of hES cells, were used in our experiments. The cells (2A,XY) were stained with Vybrant CM-DiI and were introduced into 28 mouse blastocysts (3.5 dpc) by microinjection into perivitellin spaces. First, the cell homing within recipient developing blastocysts was analyzed after culture for 24 hours in M16 (Sigma). Fifty-seven blastocysts with IDPSC incorporated into ICM were transferred to the uterus of five foster mothers. Nine fetuses were formed and removed at days 11 and 18. Thin whole-mouse frozen sections (5μm) were prepared from the fetuses obtained using a routine protocol. Fetuses were analyzed using conventional histological technique, immunofluorescence and FISH with human Y chromosome probe. The homing of IDPSC within mouse tissues was assessed with a confocal microscope (LSM 510, Zeiss, Jena, Germany). **Results and Discussion:** Undifferentiated IDPSC displayed fibroblast-like morphology prior to injection. Injected cells proliferated within the embryo as visualized by the growing number of Vybrant stained cells, which were not morphologically distinguishable from neighboring mouse cells. Confocal microscopy showed a predominant incorporation of IDPSC in ICM and in some cases they were also found in throphoblasts. All chimeras produced were composed of IDPSC and recipient mouse cells. In 11- and 18- day fetuses the engraftment of IDPSC was observed in almost all organs and tissues by confocal microscopy using DiI and anti-IDPSC antibody staining and FISH analysis as well. We showed that human IDPSC may survive and proliferate within the mouse blastocyst. Our data also suggest that these cells can enter into embryogenesis without impairing the development of the recipient embryo and even show a high level of homing in various organs and tissues. This indicates that ASC may respond to differentiation signals emanating from the mouse blastocyst. Thus, it seems that IDPSC may share not only similar gene expression profile but also developmental properties with ES cells. Further research will reveal whether ASC undergo true transformation or merely fuse with the host cells.

Supported by Clínica e Centro de Pesquisa Roger Abdelmassih

7.12. Human dental pulp stem cells as a potential alternative cell source for corneal epitelium transplantation

Monteiro BG[1], Gomes JAP[2], Maranduba CMC[1], Serafim RC[3], Melo GB[2], Silva MCP[4], Smith RL[4], Sant'Anna OA[5], Cerruti HF[6], Kerkis A[3], Kerkis I[1]

[1]Laboratório de Genética, [5]Laboratório de Imunoquímica, Instituto Butantan, SP, Brazil; [2]Centro Avançado de Superfície Ocular, Instituto da Visão, [4]Departamento de Morfologia e Genética, Universidade Federal de São Paulo, SP, Brazil; [3]Laboratório de Células-tronco, Clínica e Centro de Pesquisa em Reprodução Humana Roger Abdelmassih, SP, Brazil; [6]Clínica Cera, SP, Brazil

Introduction: Our group has reported the isolation of human immature dental pulp stem cells (hIDPSC) from deciduous teeth, which express mesenchymal stem cells (MSC) as well as human embryonic stem cell (ES) markers. The cells showed a normal karyotype during successive passages (25) and underwent spontaneous differentiation into neurons, smooth and skeletal muscle, cartilage and bone *in vitro*. After *in vivo* transplantation into mice, those cells showed rapid migration and engraftment into various tissues, such as liver, kidneys, lung, etc. However, no tumor formation was observed after hIDPSC transplantation. The relative ease of recovery, the *in vitro* expansion and the continuous expression of various markers characteristic of undifferentiated cells in IDPSC, justify their further exploration for clinical therapies. **Objective:** The aim of the study was to evaluate the role of hIDPSC in the reconstruction of the corneal epithelium in the rabbit eye after induction of total limbal deficiency (TLD). **Methods:** hIDPSC were cultivated according to [(1)]. TLD was induced by chemical burn in one eye of rabbits, in accordance with the Institute Butantan Ethics Committee (protocol nº 250/06). After 30 days, the opaque tissue formed was removed by superficial keratectomy. The experimental group received undifferentiated hIDPSC and the injured eyes were covered by amniotic membrane (AM), while control group only received AM. Both groups were sacrificed after 3 months and tissues formed onto the rabbit eyes were submitted to histological and immunohistochemistry study followed by confocal microscopy analysis. Transmission electron microscopy (TEM) was performed to evaluate the cornea epithelium formation. **Results:** The transparency of the cornea was improved throughout the follow-up in the experimental group. In the control group, the conjunctiva fully covered the cornea and was strongly vascularized. The histological study revealed uniformly formed corneal epithelium similar to normal ones. Interestingly, the experimental group corneas showed the presence of Browman's layers, which is a structural component specific for human. Moreover, these corneas were positively immunostained with specific antibody for hIDPSC, produced by our group, indicating the presence of these cells within the rabbit eyes. Differentiation of hIDPSC into corneal tissue was demonstrated by immunohistochemistry using antibodies against p63, integrin β1, CK18 and K3. RT-PCR using specific primers for human undifferentiated stem and corneal epithelium cells was performed to confirm these results. TEM showed a well-formed cornea with desmosomes, while other tests revealed all the cornea structural components. **Conclusions:** Our results suggest that the transplantation of hIDPSC into the injured rabbit eye due to TLD can improve the transparency of the newly formed cornea.

Reference: 1-Kerkis *et al.*, Cell, Tissue, Organs, 184:105, 2006.

Supported by FAPESP, CNPq, Clínica CERA, Genética Aplicada Ltda.

7.13 Bone augmentation using a biphasic bioceramic with and without a platelet concentrate

Lobo SE[1,2], Alves HJ[2], Kerkis I[2], Wykrota FHL[3]
[1]Departamento de Morfologia, Universidade Federal de Minas Gerais, BH, Brazil; [2]Laboratório de Genética, Instituto Butantan, SP, Brazil; [3]Hospital São Bento Cardioclínica S/A e EINCO Biomaterial Ltda, MG, Brazil

Introduction: Major bone loss represents one of the most challenging problems in health care. Due to the several disadvantages and undesirable reactions caused by the bone grafts used in these cases, the use of biomaterials has greatly increased. Among them, the calcium phosphate bioceramics are the most indicated. Recently, some authors have defended the idea that the application of a platelet concentrate in combination with some bone grafts is able to optimize bone formation. The lack of information regarding the benefits of added platelet concentrate to a bioceramic that is produced in Brazil promoted this research. **Objectives:** The aim of this study was to quantify bone formation with a bioceramic, with or without platelet concentrate added, using an animal model. **Methods:** Thirty adult male *New Zealand* rabbits were used in this study. The bioceramic used was Osteosynt® (EINCO Biomaterial Ltda., Belo Horizonte, Minas Gerais, Brazil). Animal care was according to the protocol of ethics in animal research. In each of the rabbits' femurs, critical size defects (CSD) were made using a 6-mm diameter trephine. The cortical and medullary tissues were removed and the defects were reconstructed as follows: blood clot, bioceramic, bioceramic + platelet concentrate and platelet concentrate. Animals were sacrificed at 15, 30 and 90 days post-operative. Platelet concentrate was obtained according to previously established protocols. Bone matrix, osteoblasts, osteocytes, blood vessels and other tissues formed were quantified by histomorphometry. Results were statistically analyzed using the *Kruskal-Wallis* test. **Results:** The CSD filled just with blood clot showed regeneration of adipose tissue in the medullary and part of cortical regions (MR, CR), representing a disorganized and smaller pattern of bone formation. The samples reconstructed with bioceramic showed bone formation inside and around totality of the granules, in both the CR and MR. When bioceramic was used in conjunction with platelet concentrate, no increase of the bone structures was achieved, while platelet concentrate used alone led to fat tissue formation in the MR and to a lesser extent in the CR. **Discussion:** These results are in contrast to data presented by other authors. We did not observe any advantage provided by the addition of platelet concentrate compared to those of bioceramic alone. However, it seems that platelet concentrate induced lamellar bone formation, indicating that the growth factors released by the platelets can contribute to bone maturation. The bone formation around and inside the bioceramic in the MR which is composed exclusively of adipose tissue is in agreement with the intrinsic osteoinduction concept. This means heterotopic induction of bone formation without addition of osteogenic or bone morphogenetic proteins. Even though it was observed in the MR, it is completely formed by adipose tissue. We conclude that the defects reconstructed with the bioceramic alone showed the best results.

Supported by EINCO Biomaterial Ltda.

7.14 *In vivo* germ line potential of human immature dental pulp derived stem cells in mouse

Kerkis I[1], Fonseca SAS[2], Lavagnolli TMC[1,2], Serafim RC[2], Abdelmassih S[2], Santos EJC[1], Abdelmassih R[2], Kerkis A[2]

[1]Laboratório de Genética, Instituto Butantan, SP, Brazil; [2]Clínica e Centro de Pesquisa em Reprodução Humana "Roger Abdelmassih", SP, Brazil

Introduction: Pluripotency is the capacity of stem cells (SC) to differentiate into derivatives of the three embryonic layers (EL) mesoderm, ectoderm and endoderm, and germ cells as well. Adult stem cells (ASC) are considered to have a restricted plasticity. Previously, we reported the isolation of human immature dental pulp stem cells (IDPSC), which express Oct4 and Nanog, markers of human embryonic stem cells, and which can differentiate into several cell types of the three EL [(1)]. **Objectives:** The aim of our study was to demonstrate the potential of these human cells to contribute to formation of male germ cells (GC) in mouse testis. **Methods:** Female cells (46, XX) at 7^{th} passage, which were positive for Oct4 and Nanog, were used. The cell suspension (10^5 cells) was stained with Vybrant CM-DiI and injected into the testis of a normal and/or sterilized (by gamma-irradiation) mouse (strain CD-1). Irradiation (150 rads) was performed 3 times (1 per month) and the cells were applied 1 month after the last irradiation. Control mice were injected with physiologic solution. The mice were killed 1, 5 and 9 days after injection. Thin whole-testis frozen sections (5µm) were prepared using a routine protocol. The presence of IDPSC in mouse testis was assessed by Vybrant CM-DiI, by immunofluorescence using human anti-IDPSC antibody produced by us, and by FISH for human X. The slides were mounted with DAPI. The homing of IDPSC within mouse testis was analyzed by confocal microscopy (LSM 510, Zeiss, Jena, Germany). **Results and Discussion:** Merged localization of both Vybrant CM-DiI (red) and anti-IDPSC (green) antibody staining was observed within cross-sections of seminiferous tubes (ST) in normal and sterilized mouse demonstrating the presence of IDPSC in mouse testis. All sections were investigated using double staining. One day after injection the cells were detected mainly in the Leydig and Sertoli cell compartments, while after 5 and 9 days, they were present in the spermatogenic cell compartment. The cells usually showed a cluster distribution within these compartments. Moreover, well-stained mature sperm, apparently originated from female IDPSC, with both Vybrant CM-DiI and anti-IDPSC antibody staining were seen in central part of the lumen. In all experiments the fluorescent signals were observed only in a few ST indicating the presence of IDPSC; however the majority of mouse ST were IDPSC free. In the ST of sterilized mice, less IDPSC and no stained sperm were observed after 9 days. Remarkably, male IDPSC were mainly detected in Leyding and Sertoli cell compartments and never in the lumen. Control mouse ST (without IDPSC) did not show any fluorescent signals. FISH analysis with the probes for human sex chromosomes showed their presence in mouse ST. These data suggest that IDPSC were capable of populating in cluster manner different compartments of mouse ST. Female IDPSC apparently showed higher *in vivo* germ line potential.

Reference: 1. Kerkis *et al.* Cell, Tissue, Organs, 184: 105, 2006.

Supported by Clinic and Research Center Roger Abdelmassih

7.15 Differentiation of somatic hybrid cells (SHC) obtained by fusion of embryonic stem (ES) cells and splenocytes into germ cells (GC) *in vitro*

Lavagnolli TMC[1,2], Fonseca SAS[3], Serafim RC[3], Abdelmassih S[3], Kerkis A[3], Kerkis I[1,2]

[1]Laboratório de Genética, Instituto Butantan, SP, Brazil; [2]Departamento de Morfologia, Universidade Federal de São Paulo, SP, Brazil; [3]Clínica e Centro de Pesquisa em Reprodução Humana, SP, Brazil

Introduction: Nuclear reprogramming is the functional conversion of the genetic material contained within a differentiated somatic cell to a state of developmental pluripotency or multipotency. An alternative approach to reprogram the somatic genome, instead of using the somatic cell nuclear transfer which has a low efficiency, involves the creation of hybrids between somatic cells and ES cells. Although this reprogramming strategy has generated considerable interest, it is still unknown if SHC are able to entirely reprogram their genome into the germ line. Recently, we demonstrated that mouse ES cells are able to generate *in vitro* concurrently both types of gametes: oocytes and sperm cells. **Objectives:** The purpose of the present work was to determine if mouse SHC, obtained by the fusion of ES cells and splenocytes, are capable of reprogramming their genome and completing the differentiation process into GC *in vitro*. **Methods:** We used SHC (2A, XY) previously obtained and characterized by our group. Moreover, we have proved that the X chromosome is derived from the splenocytes, confirming the reprogramming at least of this chromosome. To induce the SHC differentiation into GC, we obtained embryoid bodies (EB) by the hanging drop method and after three days, they were transferred to Neurobasal medium, supplemented with B27 and 0.1μM retinoic acid (RA- an efficient growth activator of mouse GC *in vitro*). Immunocytochemistry and RT-PCR analyses were performed to evaluate the pluripotent capacity of SHC and to confirm their differentiation into GC. **Results:** Immunocytochemistry analysis of EB was positive for Oct-4 antibody, confirming the pluripotent capacity of SHC. This pluripotent capacity was also assessed by RT-PCR, which demonstrated a positive expression for Sox-2 and Nanog (genes that are characteristic of pluripotency). Two days after the RA addition, enlarged cells, similar to GC, were observed migrating from the surface of the EB and later floating in the culture medium. During the process of differentiation, we observed cells resembling round and elongated spermatocytes and oocytes. We confirmed by RT-PCR the expression of Stella (a marker for primordial germ cell- PGC), Dazl (expressed in GC), Piwil 2 (a marker for PGC and spermatogonia) and other genes such as Text14, Haprin, Rnf17, Acrosin, Scyp3 and Stra-8, which are expressed in early and late stages of male GC development. We also observed the expression of ZP2 and ZP3, which represent a developmentally regulated set of genes whose expression serves as markers of mouse oocyte growth and differentiation. Furthermore, these findings were confirmed by immunocytochemistry for Fragilis, Vasa, Dazl and EMA (GC markers). **Discussion:** The ability of cells to fuse and influence each other's genome has been widely used for reprogramming somatic cells, since the hybrids obtained preserve genetic markers from both cell types. The data presented demonstrate that somatic cells were reprogrammed by the ES cell genome, allowing their differentiation into PGC *in vitro* and that these cells undergo further differentiation into GC.

Supported by FAPESP

7.16 Storage pools of proliferating cell nuclear antigen (PCNA) in the nucleus of *Trypanosoma cruzi*

Calderano SG[1], Haa VSJ[2], Rubin-de-Celis SS[2], Dossin FM[2], Mortara RA[2], Schenkman S[2], Elias MC[1]

[1]Laboratório de Parasitologia, Instituto Butantan, SP, Brazil; [2]Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, SP, Brazil

Introduction: Proliferation cell nuclear antigen (PCNA) is a homotrimeric protein that associates around double stranded DNA to form a ring like structure. PCNA interacts with DNA polymerases, enhancing the processivity of DNA replication at the fork. PCNA also serves as an anchor point for proteins that are involved in DNA replication, repair, and recombination as well as proteins involved in the regulation of the cell-division cycle. **Objective:** Here, we identified the PCNA gene from *Trypanosoma cruzi,* the etiological agent of Chagas disease, and characterized its expression and localization during the cell cycle of this protozoan parasite. **Results:** PCNA-encoding mRNA is observed throughout all the cell cycle but increases during S-phase and then decays to the basal level at the time of cell division. The same variation occurs for the protein, which is always found in the nuclear space. At the beginning of S phase, PCNA is constrained in two opposed domains surrounding the central nucleolus. When DNA replication is maximal, PCNA is constrained to the nuclear periphery, dispersing throughout all the nuclear space when replication is complete. Replication sites, as observed by BrdU incorporation, localize at the nuclear periphery. **Discussion**: These data indicate that there are storage pools of replication factories at the nucleolar periphery. During DNA synthesis there is an increment in PCNA amounts together with the transition of PCNA molecules to nuclear periphery.

Supported by FAPESP

7.17 Characterization of the putative pre-replication complex component Orc/Cdc6 of *Trypanosoma cruzi*

Godoy PDM[1], Nogueira LAJ[1], Mora AJC[2], Schenkman S[2], Elias MC[1]
[1]Laboratório de Parasitologia, Instituto Butantan, SP, Brazil; [2]Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, SP, Brazil

Introduction: The assembly of pre-replication complexes in eukaryotes begins with the binding of an origin recognition complex (ORC_{1-6}) to chromatin. Chromatin-bound ORC recruits Cdc6 and Cdt1. Cdc6 stabilizes the binding of ORC and allows Cdt1 to load the hexameric Mcm complex, whose helicase activity is essential for replication. The assembly of the pre-replication complex occurs in G1. In order to ensure that DNA molecules will be replicated only once in each cell cycle, Orc1 is degraded upon entry into S phase and re-accumulates after mitosis. In yeast, however, all six Orc subunits remain in a complex bound to replication origins throughout the entire cell cycle. Very little is known about DNA replication in trypanosomes. Unlike other eukaryotes, trypanosomes do not contain sequences in their genome that could code for ORCs, Cdc6 or Cdt1. Instead, these parasites contain one open reading frame homologous to Orc1 and Cdc6 similar to Archaea. **Materials and Methods**: Here, the Orc/Cdc6 encoding gene from *T. cruzi* was cloned and expressed in *E. coli*, and the recombinant protein was used to immunize animals. **Results and Discussion**: The anti-Orc/Cdc6 serum recognized a band of expected molecular mass in extracts of *T. cruzi* in replicative epimastigote forms. The Orc/Cdc6 localizes in the nucleus and this nuclear localization is not dependent on cell cycle phase, since cells in G1/S and G2 and even mitotic cells contain Orc/Cdc6 in the nucleus. We have previously shown that replication sites are located at the nuclear periphery of epimastigote cells [(1)]. Indeed, Orc/Cdc6 is constrained at the nuclear periphery in most replicative cells and is dispersed through the entire nuclear space of the infective forms. These data point to the role of Orc/Cdc6 in the replication of trypanosomes.

Reference: 1. Elias *et al*., Eukaryot Cell., 1:944, 2002.
Supported by FAPESP

8. Others

8.01 Mechanisms involved in antinociception induced by the C-terminal of the murine S100A9 protein on experimental neuropathic pain

Paccola CC[1,4], Gutierrez VP[1], Alves AS[3], Juliano MA[2], Juliano L[2], Britto LRG[3], Giorgi R[1]

[1]Laboratório de Fisiopatologia, Instituto Butantan, SP, Brazil; [2]Departamento de Biofísica, Instituto de Farmacologia, Universidade Federal de São Paulo, SP, Brazil; [3]Laboratório de Neurobiologia Celular, Departamento de Fisiologia e Biofísica, Instituto de Ciências Biomédicas, [4]Departamento de Patologia Experimental e Comparada, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brazil

Introduction: A synthetic peptide identical to the C-terminal of murine S100A9 protein (mS100A9p) induces antinociception in experimental acute inflammatory pain models [(1)]. Also, hyperalgesia, allodynia [(2)], and spontaneous pain in rats submitted to chronic constriction injury (CCI), a model of experimental neuropathic pain, were blocked by this peptide at day 14 after surgery. In this model, hyperalgesia is already detected at day 7 after the surgery procedure. At this time, inflammatory response is the most important component of the pain phenomena, and mS100A9p reversed the hyperalgesia stage. **Objectives:** The aim of the study was to determine the possible mechanisms involved in the antinociceptive effect of mS100A9p. The participation of opioid, 5-HT, or α_2-adrenergic receptors was evaluated at day 14 after CCI. Moreover, immediate early genes Egr-1 and Fos (cellular activity markers), as well TNFα (pro-inflammatory cytokine) expression were analyzed in the lumbar spinal cord. **Methods:** For CCI, male Wistar rats were anesthetized with halothane. Neuropathic pain was induced by four ligatures tied loosely around the right sciatic nerve as described by Bennett and Xie [(3)]. Pain behaviors were evaluated by paw pressure test [(4)] and by von Frey hairs [(5)] at day 14 after CCI. Naloxone (1µg/rat), an opioid receptor antagonist, was injected by the intraplantar route (i.pl.) immediately before intrathecal (0.5µg/rat), oral or intraplantar (8µg/rat) administration of the mS100A9p. Methysergide (5mg/kg), a 5-HT receptor antagonist, and yohimbine (1mg/kg), an α_2-adrenergic receptor antagonist, were injected by the intraperitoneal or intravenous route, respectively, before peptide i.pl. administration. Hyperalgesia and allodynia were determined 1 h after treatments. Immunohistochemistry assay was employed at days 7 and 14 after the surgery procedure to evaluate Egr-1, Fos, or TNFα expression in the spinal cord of rats submitted to CCI and treated with mS100A9p i.pl. The left side of the spinal cord (contralateral) was used as control. **Results:** The antinociceptive effect of mS100A9p, administered by different routes, was not blocked by naloxone. Similarly, methysergide and yohimbine did not modify hyperalgesia and allodynia induced by CCI. Immunohistochemical results showed that CCI increased Egr-1, Fos and TNFα expression in the spinal cord compared with contralateral side. On the other hand, mS100A9p administration at days 7 or 14 after CCI, reduced this protein expression. **Discussion:** These results demonstrate that peripheral opioid, 5-HT, or α_2-adrenergic receptors did not participate of the antinociceptive effect of mS100A9p. Furthermore, this peptide interferes with Egr-1, Fos and TNFα expression in the lumbar spinal cord.

References: 1. Dale *et al.*, Peptides, 27: 2794, 2006; 2. Paccola *et al.*, Memórias do Instituto Butantan, 2005; 3. Pain, 33: 87, 1988; 4. Randall & Selitto, Arch. Intern. Pharmacodyn., 111: 209, 1957; 5.; Chaplan *et al.*, J. Neurosci. Meth., 53: 55, 1994.

Supported by FAPESP, Fundação Butantan

8.02 Behavioral effects of a peptide isolated from β-casein chain and a possible involvement of opiate pathway

Chaguri LCAG[1], Correa GB[2], Simokomaki K[1], Lebrun I[1]
[1]Laboratório de Bioquímica e Biofísica, Instituto Butantan, SP, Brazil; [2]Universidade Adventista de São Paulo, SP, Brazil

Introduction: Biologically active peptides have been identified in several foods, such as casomorphins which participate in the neurotransmission to the brain function that could play a role in many physiological systems. The peptide YPVPQFTE derived from casein demonstrated drift cardiovascular effects and could influence behavioral parameters of rats by the peptidergic pathways linking with classical neurotransmitters such as dopamine. **Objectives:** Biologically active peptide, YPVPQFTE, from β-casein chain was investigated by behavioral analysis of rats to identify a possible role in brain function. **Methods:** Groups of rats were maintained from 21 until 70 days old in standard polypropylene cages (SC), or in an enrichment environment (EE), in the same kind of cages but with a PVC tube added as the enrichment tool. After this period, the animals were submitted to open-field and elevated plus-maze tests. The drugs used to discriminate behavior were saline, diazepam (5mg/mL) or the peptide YPVPQFTE (30μg/Kg). ANOVA was performed in all groups on the behavioral parameters in the open-field: locomotion (LO), rearing frequency (Re) and immobility posture (I) and in elevated plus-maze: % open arms entry (%E), % time spent in open arm (%T), total number of open arms entry (TN). When statistical significance occurred, Tukey-Kramer multiple comparisons test was carried out. **Results:** When analyzing the influence of the environmental condition in the behavioral tests, the group maintained in EE showed lower indices of LO and Re in the open-field and consequently higher I for the saline and diazepam assays. The peptide group showed no statistical differences in behavioral parameters in open-field test compared to the environmental condition. When analyzing the influence of the different drugs on the environmental condition, the groups administered diazepam and with peptide SC group showed higher LO frequency than the saline EE group. The saline SC and peptide EE displayed higher Re than the diazepam or saline EE groups and the immobility posture was significantly higher in diazepam EE and SC groups and in peptide EE group than the saline SC group. In the elevated plus-maze test evaluation, no differences occurred between the groups maintained at different environmental condition adopted but the pharmacological screening revealed that diazepam SC and EE group showed higher %E than the saline group SC. In %T parameter, diazepam SC group had a higher frequency than the other groups. The TN demonstrated no differences between groups under different environmental condition or pharmacological screening. **Discussion:** The peptide YPVPQFTE was isolated after tryptic hydrolysis of casein and showed a significant hypotensive effect that was not related to the angiotensin converting enzyme (ACE) but with oligopeptidase 24:15, which is a typical brain peptidase. From these aspects, behavioral events could be directly affected. Besides the previously described effect on arterial blood pressure of rats, this work showed that behavioral events could be directly affected by this peptide, since the peptide assays showed similar parameter values in the open-field test (LO, Re and I) compared to the environmental condition adopted.

Supported by Fundação Butantan

8.03 Effects of casein and soy protein on newborn Wistar rats: physical and neurobehavioral development

Mazzeo A[1], Pedicino CA[2], Dorce VAC[2], Lebrun I[1]
[1]Laboratório de Bioquímica e Biofísica, [2]Laboratório de Farmacologia, Instituto Butantan, SP, Brazil

Introduction: Casein is a source of biologically active peptides which are released by enzymatic digestion. The peptides released show several activities, such as sleep induction, growth factors and neurotransmitter release. **Objectives:** The aim of this work was to evaluate behavioral and physical development of newborn rats after treatment with casein, soy protein or saline plus 10% glucose (control). **Methods**: The newborn evaluation considered physical development (the opening of the eyes and acoustic meatus, ear unfolding, tooth eruption and testicle descent for males and vagina opening for females) and neurobehavioral observation on clinging palm, postural reflex, negative geotaxis and activity box. Three experimental groups (10 mice per group) were treated with saline plus 10% glucose, casein or soy protein. **Results**: No significant differences were observed in the physical development variables. However, mice treated with casein had a greater weight gain, and those treated with soy protein showed best results on negative geotaxis test. **Conclusion**: According to these results, we can conclude that casein stimulates muscle development, while soy protein seems to stimulate the animal motor activity.

Supported by Fundação Butantan

8.04 Purification of plasmatic factor VIII by anion exchange and gel filtration chromatography

Jinzenji D, Carvalho RR, Rodriguez ES, Raw I, Cheng E, Martins EAL
Centro de Biotecnologia, Instituto Butantan, SP, Brazil

Introduction: Brazil consumes large amount of blood derivatives, but has to import most of them. About half of the 80 million dollars/year spent to purchase blood derivatives is for hemophiliac patients. Hemophilia A is a life-threatening, disabling disorder, characterized by lack of Factor VIII (FVIII) activity. It affects 6,000-12,000 patients in Brazil. Treatment consists of infusions of FVIII obtained either by purification from plasma or as recombinant protein, none of which is produced in Brazil. Classical production of FVIII is based on Cohn's method which separates this factor from plasma cryoprecipitates and requires the use of cold rooms and centrifuges. It is not considered an efficient method concerning either purity or yield. Direct plasma chromatography is being proposed for FVIII production, as a more efficient method with consequent lower final costs. **Objectives:** Use of gel filtration in the initial step is a new validated method for FVIII purification. It is a better method when compared to Cohn´s, but it is slow and has a small capacity. We propose ion exchange chromatography prior to gel filtration. Our objectives were to: **1-** develop plasma protein purification technologies using primarily anion exchange chromatography followed by gel filtration and compare yield, quality and costs of the process; and **2-** produce antibodies to recombinant FVIII protein fragments to follow FVIII features during purification steps, through immunoblotting assays. **Methods: 1**. Chromatography: plasma is used directly for anion exchange chromatography, and the FVIII-containing eluted fraction is applied to gel filtration. Other proteins of medical interest, such as Protein C, are being examined. Analyses are made by chromogenic tests and Western blotting. **2**. Polyclonal anti-FVIII antibodies: three fragments of human FVIII gene were chosen to be cloned in *E. coli*. The expressed recombinant proteins were purified by metal ion affinity chromatography and used for antibody production in mice. **Results: 1.** FVIII is usually associated with Von Willebrand factor. In direct plasma gel filtration chromatography (using Sepharose 4FF column), FVIII activity is recovered mostly in the void volume. Using anion exchange chromatography (Q-Sepharose FF) followed by gel filtration, FVIII activity was present in lower molecular weight fractions. The same results were obtained using columns of different lengths. Substituting Sepharose 4FF with Sephacryl S-200 resulted in similar elution profiles, also indicating the recovery of FVIII in smaller complexes. **2.** Fragments of human FVIII gene were cloned, expressed and purified, and are being used for mouse immunization. **Discussion:** The proposed chromatographic sequence enables the processing of larger amounts of plasma in a shorter time. Elution patterns suggest separation of Von Willebrand factor from FVIII during anion exchange in contrast with direct gel filtration. A complete analysis of the presence of Protein C, factor IX, factor X and Von Willebrand in the elution fractions from gel filtration and anion exchange chromatography is being pursued in order to improve FVIII purification, as well as the separation of these proteins for clinical use. Antibodies will be used to determine the stability of FVIII in the purification steps.

Supported by Fapesp, CNPq, FUNDAP, Fundação Butantan

8.05 SP-A and α_1–antitrypsin (α_1-AT) porcine proteins for animal model of lung injury

Kubrusly FS[1], Dias SC[1], Sakauchi D[2], Precioso AR[3], Iourtov D[4], Veiga LR[4], Horton DSPQ[2], Piazza RMF[5], Mascaretti RS[3], Rebello CM[3], Raw I[1]

[1]Centro de Biotecnologia, [2]Serviço de Controle, [4]Serviço de Surfactante, [5]Laboratório de Bacteriologia, Instituto Butantan, SP, Brazil; [3]Departamento de Pediatria Experimental da Faculdade de Medicina, Universidade de São Paulo, SP, Brazil

Introduction: Surfactant converts large highly surface-active aggregates to smaller less active aggregates during its life cycle. This process is regulated by a serine protease (surfactant convertase) produced by type II cells and alveolar macrophages, and depends on repeated expansion and contraction of the air–fluid interface. Surfactant convertase is sensitive to inhibition by α_1–antitrypsin (α_1-AT). The addition of α_1-AT to surfactant improves its *in vivo* function and preservation of large surfactant aggregates by inhibiting the surfactant convertase. α1-AT and surfactant protein-A (SP-A) are major glycoproteins in the alveolar spaces of human lungs. These glycoproteins belong to two systems of the lung that are supposed to act independently: the surfactant system and the proteinase/proteinase inhibitor system. Evidence for possible links between the two systems does exist [(1)] and this interaction could have potential implications in the physiologic regulation of α1-antitrypsin activity, in the pathogenesis of pulmonary emphysema, and in the defense against infectious agents or in other lung injuries such as diffuse alveolar damage (DAD). **Objective**: Our interest was to provide these two purified glycoproteins to design experiments *in vitro* and *in vivo* to study the nature and consequences of such an interaction in an animal model. **Methods:** Purification protocols were developed using porcine pulmonary extracts as source of pure glycoproteins. For protein characterization, biochemical and immunological assays were performed. **Results and Discussion**: The pure porcine SP-A obtained using tangential-flow filtration followed by ion-exchange chromatography showed a mannose-binding ability that could be representative of its functionality. α_1-AT purification was obtained after acid precipitation and affinity chromatography. Porcine α_1-AT cross-reacts with human α_1-AT. **Conclusions:** Our purification protocols and characterization analysis were able to provide pure porcine SP-A and α_1-AT to be tested in pig models of lung injury since this model is considered ideal for studying human lung injury.

Reference: 1. Gorrini *et al.* Respir. Res. 6: 146, 2005.

Supported by FAPESP, CNPq, Fundação Butantan

8.06 Partial purification of prothrombin from *Bothrops jararaca* plasma

Morais KB[1,2], Sano-Martins IS[1,2], Tanaka-Azevedo AM[1]
[1]Laboratório de Fisiopatologia, Instituto Butantan, SP, Brazil; [2]Departamento de Fisiologia, Instituto de Biociências, Universidade de São Paulo, SP, Brazil

Introduction: Prothrombin is the most abundant of the vitamin K-dependent blood clotting proteins, circulating at plasma concentrations between 1 and 2 μM [(1)]. Prothrombin is the precursor of the enzyme thrombin, a serinoprotease enzyme that plays an extraordinarily prominent role in the blood coagulation and wound healing mechanisms. **Objectives**: The aim of this study was to purify *Bothrops jararaca* (*B.jararaca*) prothrombin and to compare it with human and other animals' prothrombin. **Methods**: This protein was obtained through barium chloride *B. jararaca* adsorbed plasma, two sequential steps of ammonium sulfate fractionation (35 and 70% saturation) and HiTrap DEAE Fast Flow chromatography, followed by affinity chromatography on Chelating Cu^{2+} and gel filtration on Sephacryl S-200. Along all the purification steps protein concentration was determined by absorbance at A_{280}. Amidolytic thrombin activity was measured using chromogenic substrate (S-2238) after prothrombin activation by *Oxyuranus scutellatus scutellatus* venom. **Results and discussion**: Our SDS-PAGE results suggest that the molecular mass of *B.jararaca* prothrombin is 80 kDa, which is comparable to human prothrombin, with a molecular mass of 73 kDa, including its proteolytic coagulant activity. The perspectives for this work are to improve the purification process to obtain higher purity protein and make biological and biochemical comparisons with prothrombin of other animals.

Reference: 1. Degen et al., 1983
Supported by CAPES, FAPESP

8.07 Purification of *Bothrops jararaca* antithrombin

Tanaka-Azevedo AM[1], Figueiredo AL[1], Vieira CO[1,2], Morais KB[1,2]
[1]Laboratório de Fisiopatologia, Instituto Butantan, SP, Brazil; [2]Departamento de Fisiologia, Instituto de Biociências, Universidade de São Paulo, SP, Brazil

Introduction: Antithrombin (At) is a plasma glycoprotein composed of a single chain with molecular mass about 60 kDa which is synthesized in the liver. At is an important regulator of blood coagulation not only due to its ability to inhibit thrombin, factor IXa, and factor Xa in plasma, but also to inactivate the other intrinsic coagulation pathway serine proteases such as factors XIa and XIIa. In addition, At can inactivate some noncoagulation serine proteases, such as plasmin, kallikrein and the complement enzyme C1. **Objectives**: The aim of this work was to purify *Bothrops jararaca* (*Bj*) At and to compare it with other antithrombins described. **Methods**: *Bj* plasma was diluted in 0.1 M Tris, 0.01 M citrate, 0.25 M NaCl, pH 7.4, buffer and applied to a Hi Trap Heparin HP column. The proteins were measured by absorbance at 280 nm. At activity was measured after addition of an excess of heparin and thrombin. The thrombin splits off p-nitroaniline (pNA) from the substrate H-D-Phe-Pip-Arg-pNA (S-2238), which is measured spectrophotometrically at 405 nm. The purified *Bj* antithrombin was analyzed by SDS-PAGE. **Results and discussion**: The molecular mass of *Bj* antithrombin is 70 kDa, which is comparable to human antithrombin. This purification process yielded a high purity protein. The perspective for this work is to characterize *Bj* At biologically and biochemically and to compare it with antithrombin of other animals.

Supported by FAPESP, CNPq, CAPES

8.08 Description of proteins from the saliva of *Amblyomma cajennense* tick (Acari: Ixodidae) (Fabricius: 1787) related to blood coagulation and extra cellular matrix

Simons SM[1], Oliveira DGL[1], De-Sá-Júnior PL[1], Faria F[1], Silva-Ricci ME[1], Valente RH[2], Leon IR[2], Perales J[2], Chudzinski-Tavassi AM[1]

[1]Laboratório de Bioquímica e Biofísica, Instituto Butantan, SP, Brazil; [2]Departamento de Fisiologia e Farmacodinâmica, Instituto Oswaldo Cruz, FIOCRUZ, RJ, Brazil

Introduction. Proteins isolated from the saliva of hematophages are useful tools for the understanding of many physiological processes. **Objectives**. In the present study, we characterized the cytotoxic and anti-coagulant activities of proteases and inhibitors present in the saliva *of Amblyomma cajennense* tick. **Methods**. The profile of saliva proteins was analyzed by 2D electrophoresis followed by mass spectrometry or, alternatively, by ion exchange chromatography in a FPLC system. Fractions from the chromatographic steps were investigated in regard to hemostatic parameters (coagulation, fibrinolysis and platelet aggregation) and cell survival. Samples were also incubated with several purified proteins (fibrinogen, FX, FXa, FII, thrombin, plasminogen, lysozyme, collagen, BSA, plasmin and urokinase) and submitted to SDS-PAGE. **Results**. Preliminary results demonstrated that the saliva induces fragmentation of fibrinogen and plasminogen, and forms a complex with FXa. A total of 110 spots were revealed with 2D electrophoresis (pI between 3.5 and 9.5 and MW from 12 to 160 kDa) and were analyzed by MS-MS. Ion exchange chromatography resulted in seven protein peaks (I-VII) which showed independent activities on coagulation (inhibition of platelet aggregation induced by collagen I, FXa and thrombin inhibition) and cell cytotoxicity (HUVECs, B16F10 and MIA PaCa-2). **Discussion.** The proteins discovered in this work may have therapeutic applications. The study of their structure and mechanisms of action will also clarify the mechanisms of adaptation to the host during evolution.

Supported by FAPESP, FINEP, CNPq

8.09 Protein digestion in the scorpion *Tityus serrulatus*: isolation of a major cisteine proteinase

Fuzita FJ, Lopes AR
Laboratório de Bioquímica e Biofísica, Instituto Butantan, SP, Brazil

Introduction: Scorpions are ancient chelicerates that have changed little since the Silurian and are considered the oldest known terrestrial species, which makes this species a model for the study of digestion evolution in Arthropoda. Despite their biological importance, only two enzymes involved in prey digestion are known in this group: an amylase and a lipase. Scorpion digestive glands, called hepatopancreas, occupy most of the abdominal region. **Objective**: We aimed to characterize protein digestion in scorpions. **Methods**: In order to characterize protein digestion in scorpions, *Tityus serrulatus* hepatopancreas was isolated and homogenized. Protease activity was determined using hemoglobin, casein-FITC and Z-FR-MCA (carbobenzoxy-Phe-Arg-7amido4methyl coumarin) as substrates. Pepstatin, E-64, PMSF, and phenanthroline were used as inhibitors allowing the classification of proteolytic activity. **Results**: Low activity was observed on casein-FITC. The major proteolytic activity on hemoglobin and Z-FR-MCA shows that acidic activation is completely inhibited by E-64 and is also dependent on EDTA. Anion exchange chromatography indicates the presence of at least three cysteine-proteinases involved in protein digestion in *Tityus serrulatus* (yield: 600%). These activities demonstrated an acid pH optimum (3.0, 4.0 and 5.4), molecular weight of 60 kDa (non-activated sample) and 44 kDa (activated sample). Partial isolations of these enzymes were obtained with Arginine-Sepharose affinity chromatography (yield: 150%, purification factor: 8x). Samples of a pool from a combination of affinity, anion exchange chromatography and gel filtration applied to a 12% polyacrylamide gel showed the enrichment of a protein band that has cysteine endopeptidase activity and confirms the expected molecular mass estimated by the gel. Kinetic characterization using different substrates (Z-FR-MCA and Z-RR-MCA) indicates that this enzyme is a cathepsin-L-like enzyme. **Discussion:** The results obtained in the characterization of protein digestion in *Tityus serrulatus* indicate that the main endopeptidase is a cysteine endopeptidase. These results are in agreement with results reported in the literature for other Arachnida groups such as ticks, which also have a cysteine endopeptidase involved in digestion.

Supported by FAPESP

8.10 Characterization of digestive carbohydrases from the scorpion *Tityus serrulatus*

Moreti R, Lopes AR
Laboratório de Bioquímica e Biofísica, Instituto Butantan, SP, Brazil

Introduction: Scorpions are ancient chelicerates that have changed little since the Silurian and are considered the oldest known terrestrial species, which makes this species a model to the study of digestion evolution in Arthropoda. Scorpion digestive glands, called hepatopancreas, occupy most of the abdominal region. **Objectives:** We aimed to characterize carbohydrate digestion in scorpions. We determined amylase, trehalase, α-fucosidase, β-glucosaminidase, chitinase, α-glucosidase, α-galactosidase activities in the hepatopancreas from *Tityus serrulatus*. Properties such as pH effect, molecular mass, separation on anion-exchange chromatography and Km were determined. **Methods:** *Tityus serrulatus* hepatopancreas was isolated and homogenized in Milli Q water. The following substrates were used to measure enzyme activities: amylase (starch), trehalase (trehalose), chitinase (4-methylumbelliferyl-β-N',N",N'"-triacetylchitotrioside,MUC3), β-glucosaminidase (4-methylumbelliferyl-β-N'-acetylglucosamine, MUNAG), α-glucosidase (4-methyl-umbelliferyl-α-glucoside), α-fucosidase (MUαGlu4-methyl-umbelliferyl-α-L-fucoside), α-galactosidase (4-methylumbelliferyl-α-D-galactoside). Gel filtration chromatography was used to determine the molecular mass of these activities. Hitrap Q columns using Tris-HCl 20 mM pH 7.0 buffer and a NaCl gradient (0-1 M) were used in anion exchange separation of these activities. The buffers 0.1 M citrate-phosphate (2.5 – 6.0), 0.1 M MES (6.0 – 6.5), 0.1 M Tris-HCl (7.0 – 9.0) and 0.1 M Gly-NaOH (9.0 – 10.0) were used to determine the effect of pH on carbohydrases activities. **Results:** We determined carbohydrases present in the hepatopancreas (Hp) from the scorpion *Tityus serrulatus*. In Hp, the following enzyme absolute and specific activities were determined: amylase 17 U/gut (0.8 mU/μg); trehalase 1.3 U/gut (0.07 mU/μg); chitinase 3500 fluorescence units/min/gut (0.09 FU/ug); α-glucosidase 240 FU/min/gut (0.009 FU/ug); α-galactosidase 2.0 FU/min/gut ($9.8x10^{-5}$ FU/μg); β-glucosaminidase 9300 FU/min/gut (0.4unF/μg); α-fucosidase 3500 FU/min/gut (0.14 FU/ug). Amylase showed a molecular mass of 53.1 kDa, at least two different activities separated by anion-exchange chromatography and four different pH optima (3.5, 4.5, 5.9 and 7.8). Trehalase showed a molecular mass of 60 kDa, and only one peak of activity was observed after anion-exchange chromatography. Preliminary results indicate acidic activity for this trehalase, with a Km of 3.2 mM. Chitinase showed a predominant molecular mass of 76.6 kDa and two different activities were identified after anion-exchange chromatography. The study of pH effect indicates at least two activities (pH optima of 5.8 and 7.3). α-Glucosidase displayed a molecular mass of 60 kDa. α-Galactosidase had two different activities on gel filtration, with a molecular mass of 67.8 kDa and 41.6 kDa. β-Glucosaminidase showed a molecular mass of 76.6 kDa and two different activities were identified after anion-exchange chromatography. Preliminary results indicated acidic activity. α-Fucosidase demonstrated a molecular mass of 86.5 kDa and a pH optimum of 5.0 and, preliminary results indicate two different activities on anion-exchange chromatography. **Discussion:** The main digestive carbohydrase activities were identified in samples of *Tityus serrulatus* Hp, and fucosidase, chitinase and glucosaminidase were found to be the most active enzymes.

Supported by FAPESP

8.11 Effect of the ovariectomy on the expression of muscarinic acetylcholine receptor subtypes in the rat hippocampus

Cardoso CC[1], Porto CS[2], Abdalla FMF[1]
[1]Laboratório de Farmacologia, Instituto Butantan, SP, Brazil; [2]Seção de Endocrinologia Experimental, Departamento de Farmacologia, Universidade Federal de São Paulo, SP, Brazil

Introduction: Molecular cloning studies have revealed the existence of five distinct mammalian muscarinic acethylcholine receptor (machr) subtypes (m_1-m_5). Machr subtypes are expressed differently in the rat hippocampus, and often, more than one subtype is present in one cell. Studies have shown that estrogen affects machrs in different rat brain areas. The mechanism underlying the effect of estrogen on cholinergic function of basal forebrain has not been well elucidated, and may be related to changes at the machr levels. In our laboratory, we have shown that there is an increased density of machrs in rat hippocampus 15 days after ovariectomy when compared to the proestrus rat (control) [(1)]. **Objectives:** The present study was designed to investigate the effect of ovariectomy on the expression of m_1, m_2 and m_3 machr subtypes in rat hippocampus. **Methods:** The hippocampus was obtained from rats in proestrus (control) (ct) and ovariectomized 15 days prior (c15). Hippocampus membrane was incubated with [^{3}h]quinuclidinyl benzylate ([^{3}h]qnb) (20-100 fmols) in the presence of primary m_1, m_2 and m_3 subtype-specific antibodies (h-120, sc-9106, 1:1000; h-170, sc-9107, 1:1000 and h-210 sc-9108,1:1000, respectively, m_1, m_2 and m_3, Santa Cruz Biotechnology, CA, USA) (total) and IgG (nonspecific)(4 hr/4^{o}c). Protein levels of m_1, m_2 and m_3 machr subtypes were performed by immunoprecipitation assay as previously described. **Results:** c15 rats increased the binding of [^{3}h]qnb when compared with those obtained from ct animals (respectively, 1056.52 ± 115.58, n=4 and 553.88 ± 16.47 fmol, n=7), indicating an increase in the density of machrs as previously reported. The percentage of immunoprecipitation to m_1, m_2 and m_3 were similar between ct and c15 animals (respectively, 61.18 ± 5.75, n=6 and 62.25 ± 3.22 %, n=4 to m_1, 26.67 ± 3.33, n=3 and 20.83 ± 8.56 %, n=6 to m_2 and 9.50 ± 2.50, n=3 and 5.80 ± 1.74 %, n=3 to m_3). **Discussion**: The results provide evidence that ovariectomy does not change the expression of m_1, m_2 and m_3 machr subtypes in the rat hippocampus.

Reference: 1. Cardoso *et al.*, Neuroendocrinol., 80: 379, 2004.
Supported by Fapesp, CNPq

8.12 Study of the neurochemical modifications induced by age on levels of cathecholamines and metabolites in the rat vas deferens

Freitas TA[1], Liria BF[2], Bellot RG[2], Conceição IM[1]
[1]Laboratório de Farmacologia, Instituto Butantan, SP, Brazil; [2]Faculdade de Ciências Biológicas e da Saúde, UMESP, SP, Brazil

Introduction: Vas deferens is supplied with a dense sympathetic innervation, with noradrenaline as their main neurotransmitter. Sympathetic neurons begin to emerge early in the neonatal period and their maturation start in the second week of life. The density of innervation increases rapidly until the tenth day of life, which is followed by a slower period until the sixth month. The analysis of neurotransmitter contents of the vas deferens of neonate and aged animals is rare and almost always limited to noradrenaline content. **Objectives:** The aim of present study was to determine the effects of age on the levels of noradrenaline (NA), its precursor, dopamine (DA), and its metabolite VMA, in rat vas deferens. **Methods and Results:** Male Wistar rats aged 0 (neonates), 1, 3, 6 and 24 months (n=10/group) were used in the study. The vas deferens was removed and processed for the measurement of neurotransmitters in an HPLC system coupled to an electrochemical detector. Our results show that the NA content in neonates (671.1±131.1 pmol/mg tissue) was greater than that of 1-month-old (39.9±15 pmol/mg tissue), 3-month-old (71.8±24.2 pmol/mg tissue), 6-month-old (145.6±33.3 pmol/mg tissue) and 24-month-old (148.9 ±35.2 pmol/mg tissue) animals. DA values were greater in neonates (41.1±20.3 pmol/mg tissue) when compared to animals 1 month-old (12.3±1.8 pmol/mg tissue). **Discussion:** Our data indicate that in the rat vas deferens of neonates, there is a very high content of NA when compared with animals 1, 3, 6 or 24 months old. This result is likely related to the rapid and intense growth of sympathetic nerves observed in this period.

Supported by UMESP, Fundação Butantan, FAPESP

8.13 Nongenomic actions of 17β-estradiol and ICI 182,780 on PLC-mediated phosphoinositide hydrolysis in rat uterus

Konigame VC[1], Porto CS[2], Abdalla FMF[1]

[1]Laboratório de Farmacologia, Instituto Butantan, SP, Brazil; [2]Seção de Endocrinologia Experimental, Departamento de Farmacologia, Universidade Federal de São Paulo, SP, Brazil

Introduction: 17β-Estradiol plays an important role in the development and function of the female reproductive organs (1). In the rat uterus, the presence of estrogen receptors ERα and ERβ has been shown (2). The activation of ERs by 17β-estradiol is important in cell proliferation and differentiation. These effects can be blocked by treatment of the animals with the antiestrogen ICI 182,780 (fulvestrant) (3). In addition to the classic genomic mechanism of estrogen actions, this steroid also exerts rapid, nongenomic actions initiated at the cell surface by translocation of ERs from nuclei to the plasma membrane, and/or mediated by G-protein coupled receptor GPR30 (4). Furthermore, ICI 182,780 induces activation of GPR30 in different cell types. The intracellular mechanisms involved in the nongenomic actions of 17β-estradiol and ICI 182,780 have not been explored in the uterus. **Objectives**: The aim of the present study was to investigate the effects of 17β-estradiol and ICI 182,780 on the intracellular signaling pathway (PLC-mediated phosphoinositide hydrolysis) linked to activation of estrogen receptors in rat uterus. **Methods:** Total intracellular [^{3}H]-inositol phosphate content was measured in whole uterus, myometrium and endometrium obtained from rats in estrus, as previously described[3]. **Results:** 17β-Estradiol (1 nM) and ICI 182,780 (1 nM) caused a time-dependent (30 s to 30 min) increase in the accumulation of total [^{3}H]-inositol phosphate in whole uterus. The maximum effects were observed after 5 min (31.82 ± 3.46% over baseline level, n=4) and 1 min (17.89 ± 1.92% over baseline level, n=4) of incubation with 17β-estradiol and ICI 182,780, respectively. In the myometrium, both 17β-estradiol (5 min) and ICI 182,780 (1 min) did not change the total [^{3}H]-inositol phosphate content. However, in the endometrium, 17β-estradiol (5 min) and ICI 182,780 (1 min), respectively, induced an increase in total [^{3}H]-inositol phosphate content of 37.66 ± 7.96%, n=6 and 22.11 ± 7.15%, n=5 over baseline level. **Conclusion:** These results indicate nongenomic actions of 17β-estradiol and ICI 182,780 on total [^{3}H]-inositol phosphate content in the endometrium and suggest a role in the function of the uterus.

References: 1. Ann. N. Y. Acad. Sci., 955: 48, 2002; 2. Endocrinology, 138: 863, 1997; 3. Reprod. Biol. Endocrinol. 1: 40, 2003; 4. Mol. Cel. Endocr.160:17, 2000.

Supported by FAPESP, CNPq

8.14 Toxic effects of water samples of Tietê River in adults and embryos of *Biomphalaria glabrata* (Say, 1818)

Tallarico LF[1,2], Grazeffe V[1], Okazaki K[2], Kawano T[1], Nakano E[1]
[1]Laboratório de Parasitologia/Malacologia, Instituto Butantan, SP, Brazil; [2]Centro de Biologia Molecular, Instituto de Pesquisas Energéticas e Nucleares, CNEN, SP, Brazil

Introduction: A wide range of industrial wastes are discharged into the River Tietê in the region of Suzano. The Wastewater Treatment Plant of SABESP is responsible for biological treatment of industrial and domestic effluents. This plant works at 70% power capacity at present, and domestic effluents represent the main contribution. In addition to validating acute toxicity assays, official aquatic environmental monitoring programs have requested chronic toxicity studies with assessment of effects on fecundity, embryo-larval development, reproduction and biomass production. A good model for toxicity studies for a variety of physical and chemical agents is the freshwater snail *B. glabrata* which has a short lifespan and shows easy breeding and reproduction in the laboratory throughout the year. Besides acute toxicity, effects on fecundity, reproduction and embryo-larval development have been studied. **Objectives:** The aim of the study was evaluate the potential impact of secondary effluent discharge on Tietê River by analyzing acute toxicity in *Biomphalaria glabrata*. **Material and Methods:** Acute toxicity assays were conducted in adult specimens and embryos at the blastula, gastrula, trocophore and veliger stages of the freshwater snail *B. glabrata*. Four sites were chosen for sampling: in the river, 200 m upstream from the treatment station; the influent and effluent of the municipal wastewater system; and in the river, 200 m downstream from the treatment station discharge. **Results and Discussion:** The work was initiated in September, 2005, and at least three additional samplings were later performed. In September, 2005, the station affluent was toxic to the snail *B. glabrata* yielding the following LC_{50} values: 37.76% for adult snails and 13.99%, 20.09%, 23.15% and 23.80% for embryos at the blastula, gastrula, trocophore and veliger stages, respectively. Only the crude sample of the station effluent was toxic for embryos, but not to adult snails. Acute toxicity was remarkably reduced in August, 2006, as shown by the lower LC50 values found: 44.19%, 41.91%, 58.58% and 60.76% for embryos at blastula, gastrula, trocophore and veliger stages, respectively, and 100% for adult snails. The station effluent was not toxic to adult and embryos. Water samples in February, 2007 was toxic only to adult snails, with an LC_{50} value of 32.53%. The station effluent was not toxic to adult and embryos. In all samplings, after being discharged into the river, treated effluents were not toxic to adults and embryos of *B. glabrata.* In this study, the potential impact of effluent discharges on the biota of Tietê River was shown. Biological treatment was quite efficient in reducing toxicity to *B. glabrata* as shown by the low toxicity of the effluent plant samplings. With the dilution effect of the river, acute toxicity to *B. glabrata* was eliminated. These results show the importance of biological treatment of effluents in reducing acute toxicity.

Financial Support: FAPESP, CNPq
Acknowledgements to the technical Support of SABESP, Suzano, SP

8.15 Behaviour of opossums (*Didelphis sp*) facing physical barriers in an experimental electrical substation

Suzuki H[1], Vasconcellos TP[1], Oliveira L[1], Hingst-Zaher E[2], Cavaleiro M[3], Larizzatti SF[3], Antoniazzi MM[1], Jared C[1]

[1]Laboratório de Biologia Celular, Instituto Butantan, SP, Brazil; [2]Museu de Zoologia, Universidade de São Paulo, SP, Brazil; [3]Gerência de Meio Ambiente, Elektro, SP, Brazil

Introduction: The South American opossums (*Didelphis sp*) are among the few wild mammalian species that can adapt to environments modified by man. Due to their nocturnal and scansorial habits, they may be extremely inconvenient to humans, because they can easily introduce themselves into places inaccessible to other animals. When penetrating electric power distribution facilities, they can damage energy equipment, which can very often cause black-outs in large areas. **Objectives**: We aimed to monitor the behavior of opossums using physical barriers in an attempt to hinder their climbing into the portico or enclosure of an experimental area. **Material and Methods**: A closed experimental area of 90m^2 was constructed, having in its center a portico used in Elektro substations. The portico, measuring 5.0 m x 2.65 m, is originally formed by 4 electrical cables, 2 horizontal bars, and an X structure which diagonally fixes the bars. Specimens of *Didelphis sp* were collected in the State of São Paulo and, after a period of adaptation in the experimental area, were individually monitored by 8 cameras located in the corners and the center of each wall, from 6:00 p.m. to 6:00 a.m. As physical barriers, glass-fiber domes strategically placed on the beams with an I shape (vertical portico) and L shape (X structure) were used. In one corner of the experimental area, a 2.0-m high enclosure measuring 1.0 x 2.5 m fenced with a metal net was constructed. As a physical barrier, a 40-cm galvanized plate was welded in the upper part of this enclosure, at 45º and 1.5 m from the floor. In a simulation of "invasion" to the substation through the fence, the opossums were individually placed inside the enclosure and stimulated, by food, to overcome it. After the experiments, the animals were returned to nature, in the same place where they were captured (coordinates previously determined by GPS). **Results**: The introduction of the fenced enclosure in the experimental area was very significant and enlightening. After this introduction, the animals showed a tendency to stand at higher places, sharing their time of activity climbing both the portico structures and the enclosure fence. We also observed that some individuals spent most of time "perched" on the fence. Throughout the experiments, the opossums were observed several times at daybreak "perched" on the portico, hiding in crevices of the structure, or upon the enclosure fence. Concerning the domes, results showed that their efficiency was around 84% if their rims are placed on the vertical portico, at 20 cm, and on X structure, at 30 cm from surfaces that can be used as support for the animals. If placed at shorter distances, the opossums could easily overcome them. **Discussion**: The opossums have the natural tendency of scansoriality and do not need any type of stimulus to climb the vertical portico or the X structure. Considering the opossum behavior, we can conclude that the fenced enclosure introduced in the experimental area can be, by and large, an attractant to opossums. It is suggested to rebuild the domes taking into account the opossum body measurements since they were overcome by nearly 16% of the animals tested.

Supported by Aneel/Elektro

8.16 Populational studies of *Culex quinquefasciatus* (Culicidae, Diptera) from São Paulo, Brazil: a geometrical morphometrics approach

Peruzin MCJ, Moratore C, Devicari M, Rocha LS
Laboratório de Parasitologia, Instituto Butantan, SP, Brazil

Introduction: The mosquito *Culex quinquefasciatus* is the main vector of bancroftian filariasis in the Neotropics and is able to transmit arboviruses (i.e. WNV) among humans and other animals. In Sao Paulo County, there is a population apparently resistant to periodical applications of pyrethroid insecticides and to high levels of pollution of the Pinheiros River (RP) where it lives. In the same county, there is another population living in an urban park called "Parque Ecológico do Tietê" (PET) which is located some kilometers away from RP. In that park, there is no insecticide application, low levels of pollution and the species feed on blood of humans and migrant animals which are potential reservoirs of several parasites. Methods for controlling these mosquitoes frequently face a limiting factor: *microevolution*, the process through which these insects develop resistance to insecticides and tolerance to polluted urban environments. Therefore, research into the microevolution of Culicids is a central question in medical entomology. Microevolutionary processes may be detected by wing morphology. Since wing patterns may be influenced by geographic distance and environmental conditions, they frequently show evidence of genetic variations in a population fashion. **Objectives**: We compared morphometric variables among population samples of *Cx. quinquefasciatus* in order to: 1) examine morphological variations between PET and RP populations, possibly resulting from the presence or absence of insecticide/pollution in these locations, and 2) detect possible morphologic variations of RP population that occurred within three years (2004-2007). **Methods**: The wings of males and females of *Cx. quinquefasciatus* were mounted on a slide with coverslip and digitally photographed. Geometric morphometric analysis of wings was performed on 280 specimens from Pinheiros River edge (RP), where 152 individuals were collected in March, 2004 and 128 in May, 2007. Another 140 specimens from "Parque Ecológico do Tietê" (PET) were collected in June, 2005. For the analyses of the main geometric morphometric analyses such as digitization of 17 wing landmarks, computing wing conformational consensus, centroid size and canonical variables, we utilized the following software packs: Tps (QSC-James Rohlf), Statistica 4.3 (StatSoft) and InStat3. **Results**: The analyses of wing consensus and centroid sizes revealed slight dissimilarities (T test, $p<0.05$) between the two populations (RP and PET). The same analyses indicated that temporal variations did not occur at the RP site over three years (2004-2007), at least regarding these markers. The analysis based on the canonical variables did not show variations in any comparisons. **Discussion**: The population variations between PET and RP specimens are possibly due to different environmental conditions (i.e., insecticide use, pollution) of each region. The absence of temporal variation in RP population may indicate that demographic fluctuations were not strong and/or time was not long enough to produce detectable character variation. One interpretation of these findings is that the microevolution of wing features in *Cx. quinquefasciatus* is not detectably fast when a three-year period is considered.

Supported by FAPESP
Acknowledgements to Sumire Hibi, CCZ - São Paulo

8.17 Polytene chromosomes of Carnoy-preserved pupae *Culex quinquefasciatus* (Diptera: Culicidae)

Moratore C, Devicari M, Peruzin MCJ, Rocha LS
Laboratório de Parasitologia, Instituto Butantan, SP, Brazil

Introduction: The importance of mosquitoes to the public health system stems from the fact that many species transmit etiological agents of diseases among humans and other animals. Frequently, they play a central role in epidemics due to their highly dense populations and high dispersion velocity. Studies of polytene chromosomes (of salivary gland and Malpighian tubule) are frequently used in population research of Diptera since they bear several characters of physiological, genetic and evolutionary importance. Characters of these chromosomes such as bands and puffs have been analyzed, and have permitted, for instance, the localization and activity of esterase-family genes, which are involved with insecticide resistance. Population studies based on polytene features have also been performed in Culicidae. Studies of populations focusing on natural chromosomal variability should be performed with individuals collected in nature rather than reared in captivity. The ability to obtain polytene chromosomes from Carnoy-fixed or liquid nitrogen-frozen individuals would permit natural samples to be stored until the exact moment of preparation. **Objective:** Our objective was to test the viability of obtaining polytene chromosomes of *Culex quinquefasciatus* from Carnoy-fixed or liquid nitrogen-frozen pupae, with a cytogenetic quality similar to that obtained from fresh animals. **Methods:** We used here pupae of *Culex quinquefasciatus* collected in a polluted river of Parque Ecológico do Tietê (São Paulo, SP, Brazil - April 2007) and also liquid-nitrogen-frozen pupae/larvae of other Culicids and *Drosophila melanogaster*. The methods for polytene preparation were modified from that reported previously [(1)] and are summarized as follows: dissect Carnoy-fixed pupae in distilled water; stain in 2% orcein for 5 min; transfer to a drop of acid solution (a 1:1 mixture of lactic acid 85% + acetic acid 100%). **Results:** From Carnoy-fixed pupae, we obtained an approximate efficiency of 42% (proportion of individuals with analyzable polytenes). The polytenes obtained showed the resolution of banding and puffs of similar quality compared to those published in the literature. The polytenes obtained from frozen pupae of Culicidae were not of good quality, although those from frozen *D. melanogaster* larvae yielded good polytenes. **Discussion:** The results indicate that obtaining polytenes from fixed and frozen specimens is a viable technique. This is particularly useful for population surveys aimed at evaluating natural genetic variability, since it eliminates the necessity of processing fresh mosquitoes immediately after collecting them. We believe that in the near future it will be possible to obtain a good polytene preparation from frozen pupae of *Culex quinquefasciatus*, as we obtained with *D. melanogaster*.

Reference: 1. Campos *et al.* Mem. Inst. Oswaldo Cruz, 3: 383, 2003.
Supported by FAPESP

8.18 New morphological taxonomic markers for diagnosis of Culicids of medical importance (Diptera)

Devicari M, Moratore C, Peruzin MCJ, Rocha LS
Laboratório de Parasitologia, Instituto Butantan, SP, Brazil

Introduction. The culicids *Aedes sca*pularis, *Aedes aegypti* and *Culex* quinquefasciatus are dipterans of great interest for public health owing to the fact they are vectors of several human parasitoses. The species *Ae. scapularis* is widely distributed in the Americas, and it is typical of natural environments with little human influence although it has been also reported to live in urban areas. In addition, as a vector of arboviruses it has been involved in the transmission of the Rocio virus in state of São Paulo. *Cx. quinquefasciatus* is an anthropophilic mosquito with a pantropical distribution and a remarkable tendency for developing resistance to pollution and insecticides. *Aedes aegypti* is also anthropophilic and distributed worldwide, and it has vectorial capacity for dengue and yellow fever. The correct taxonomic identification of a species of medical interest is very important. However, its identification is jeopardized when samples are stored in ethanol, since that fixative may damage important taxonomic characters. Thus, characters that can withstand the fixation procedure should be useful in taxonomic approaches. **Objective**: We aimed to find taxonomic markers for Culicidae that can be used in cases where the sample is preserved in ethanol or where only one sex is available. **Method.** The samples of *Aedes scapularis* and *Culex* quinquefasciatus were collected respectively in Feb/1998 and Jun/2005, at Parque Ecológico do Tietê (São Paulo, SP, Brazil). The sample of *Aedes aegypti* was collected in Mar/2007 in the district of Vila Mariana in the city of São Paulo. Specimens were fixed in 70% ethanol. The wings of males and females of *Cx. quinquefasciatus* were mounted on a slide with coverslip and digitally photographed. of Geometric morphometrics methods were applied, and to obtain data such as digitzation of 17 wing landmarks and computing wing conformational consensus, centroid size and canonical variables, we utilized the following software packs: Tps (QSC-James Rohlf), Statistica 4.3 (StatSoft) and InStat3. **Results.** The analysis of wing consensus exhibited a slight difference among the three species. The centroid sizes of *Aedes scapularis* and *Culex quinquefascitus* were similar, whereas the centroid of *Aedes aegypti* was significantly smaller. The analysis of canonical variables pointed to differences among the three species. The three types of analyses showed the existence of dimorphism in the three species. **Discussion:** Geometric morphometrics has been useful for several biological models, but here it is used for culicids for the first time. Geometric morphometrics appeared to be of great taxonomic utility in demonstrating species-specific wing characters in the three species, even for ethanol-preserved samples. Moreover, from this point on, the detected wing sexual dimorphism will permit the identification of the species from a sample in which only one sex is available. We believe that such novelties can be useful in demographic/epidemiological studies. We also believe that such approach can be extended to other species of public health importance.

Supported by FAPESP

8.19 Differences in element concentrations in horse whole blood for hyperimmune serum production using NAA

Baptista TS[1,2] , Zamboni C[1] , Marcelino JR[2] , Freitas MG[2]
[1]Seção de Processamento de Plasmas Hiperimunes, Instituto Butantan, SP, Brazil; [2]Laboratório de Espectroscopia e Espectrometria das Radiações, Instituto de Pesquisas Energéticas e Nucleares, CNEN, SP, Brazil

Introduction: In the last years, we have performed several investigations in veterinary clinical medicine using a nuclear procedure, namely neutron activation analysis (NAA). This procedure was chosen because it is considered an efficient method to perform biochemical analysis of whole blood. These investigations have been carried out with success in beagles, hamsters, rabbits, rats and also in several mouse strains as well as humans. Now, we intend to extend this application to determine the concentrations of Na, K and Cl in horses used for serum production. These data are very important because these elements are major components of blood, and consequently small variation in their concentration are an important indication of the health status of theses animals during the course of the hyperimmunization process in serum production. **Objectives:** The aim of the present work was to compare the blood Na, K and Cl concentrations in hyperimmuned horses with the animals from the control group (non hyperimmuned horses) using NAA. **Methods:** For this study, twenty-five horses (race not defined) aged 12-36 months, from São Joaquim Farm at Butantan Institute (São Paulo), were used. About 0.5 mL of whole blood was collected by jugular puncture in Vacutainer tubes (vacuum plastic tubing) without anticoagulant, using of 25 x 8mm needles. Aliquots of 100 μl (prepared in duplicate) were transferred to filter paper and dried for few minutes using an infrared lamp. After that, each sample was sealed in an individual polyethylene bag and irradiated with thermal neutrons in the IEA R1 nuclear reactor at IPEN facilities. **Results**: The following arithmetic means considering one standard deviation were obtained for the control group: 1.96 ± 0.24 g/l (Na); 1.58 ± 0.39 g/l (K) and 2.41 ± 0.26 g/l (Cl) as well as for the hyperimmuned group: 2.21± 0.29 g/l (Na); 1.69 ± 0.18 g /l (K) and 2.55 ± 0.16 g/l (Cl) **Conclusion**: The data obtained in this investigation indicate an increase in the blood concentrations of Cl, K and Na for the hyperimmuned group. This information is very important because it allows a detailed study of the course of the hyperimmunization process in serum production.

8.20 Metabolic study of rhEPO-producing CHO cells in roller bottles during a 24-h cycle

Garbuio A, Inocencio AL, Targino RC, Rodrigues MTA, Moro AM
Laboratório de Anticorpos Monoclonais, Instituto Butantan, SP, Brazil

Introduction: Erythropoietin (EPO) is a hormone that regulates the production of erythrocytes in mammals. It can be obtained by the cultivation of recombinant CHO cells (CHO-EPO) in roller bottles in repeated batch mode. For process optimization, the culture medium must supply the nutritional requirements of the cells. The medium composition and the production process influence both the product quality and overall costs. As roller bottles are fed every 24 h with a complete change of the medium, thus creating a fresh medium/spent medium cycle, it is important to know the metabolic changes occurring during this period of time. From previous experience with other media, we knew that glutamine was completely depleted well before 24h. **Objectives:** The aim of this study was to access the metabolic changes associated with EPO production during a 24-h cycle, monitored hour by hour. **Methods:** Roller bottles, 850 cm², were inoculated with CHO-EPO cells in DME-F12, supplemented with 10% fetal bovine serum (FBS), and the cultures maintained at 37ºC. After monolayer cell confluence, FBS concentration was lowered to 1%. After 24 h, this medium was removed, the monolayer was washed with PBS and the following culture media were added to the roller bottles, in duplicate: (DME-F12-1%FBS, DME-F12 (Sigma), Bumel Formulation I and II (proprietary media), VPSFM (Gibco) and CHO IIIA (Gibco). After medium change, hourly sampling was started. The biochemical analyses were performed with a Bioprofile 400 Analyzer (Nova Biomedical) and the EPO concentration was measured by ELISA. **Results & Discussion:** The rollers bottles cultivated with the media DME/F12-1%FCS and VPSFM achieved the highest EPO production average rates (4.26 kUI/h and 3.37 kUI/h, respectively). VPSFM showed the highest rates of glucose and glutamine consumption, as well as the highest production rate of lactate and ammonia. Regarding carbon sources, only this medium reached critical glucose concentration (0.5 g/L) in by 24 h. Additionally, the concentration of ammonia reached the highest acceptable level for cell cultivation (5.54 mmol/L). Despite that DME-F12 medium needs serum supplementation, our experience showed that it is possible to introduce it without FBS when the cell monolayer is confluent. Nevertheless, in this study, the production of EPO was probably affected by a buffering problem, where a final pH of 7.3 was observed, unlike with other media in which the final pH ranged from 7.13 to 6.86. DME-F12 1% FBS, DME-F12 and VPSFM showed a ratio of glucose to glutamine consumption (VGluc/VGln) of 0.38, 0.50 and 0.58, respectively, whereas Bumel formulation I, II and CHO-IIIA showed a ratio of 0.9. Bumel Formulation I and II showed similar average consumption rates for glucose and glutamine and production of metabolites. However, the average EPO production rate in the Formulation I (2.02 kUI/h) was two times higher than that observed in Formulation II (1.11 kUI/h). According to the available biochemical data, there was a difference in the sodium concentration, 25% higher in Formulation I, with consequences on the average osmolarity (402 mOsm/kg for Formulation I and 327 mOsm/kg for Formulation II). The best EPO production rate was achieved with serum-supplemented medium, which demonstrates the challenge of replacing this component. Further studies are underway to evaluate product quality.

Supported by Fundação Butantan

8.21 Cloning and transient expression analysis of human glucocerebrosidase (GCR) in COS-7 cells

Novo JB[1], Morganti L[2], Magalhães GS[3], Raw I[1] and Ho PL[1]
[1]Centro de Biotecnologia, [3]Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil; [2]Centro de Biotecnologia, Instituto de Pesquisas Energéticas e Nucleares, CNEN, SP, Brazil

Introduction: Glucocerebrosidase (GCR) is a lysosomal hydrolase which degrades the glycolipid glucocerebroside to glucose and ceramide. Deficiency of this enzyme activity results in Gaucher's disease, the most common lysosomal storage disorder characterized by the accumulation of glucocerebroside in macrophages, particularly those in the liver, spleen and bone marrow. Currently, available treatment for Gaucher's disease includes enzyme replacement therapy using a recombinant form of GCR expressed in CHO cells, which has been successful in alleviating the symptoms. **Objectives:** In this work, human GCR cDNA was cloned, and the expression plasmid was functionally characterized by transient transfection before using the more laborious procedure of isolating and characterizing stable transfected cell lines. **Methods:** The GCR cDNA containing its own signal peptide or an Ig kappa-chain signal peptide were amplified by PCR, cloned in pED vector which provides a high-level expression of heterologous proteins in mammalian cells and analyzed by transient expression in transfected COS-7 cells. **Results and Discussion:** The presence of the expected protein band for the glycosylated GCR was detected by Western blotting analysis using anti-GCR antibodies prepared in our laboratory against the recombinant protein expressed in *Escherichia coli*. Thus, the expression plasmid observed by transient expression was functional. The subsequent steps include stable transfection in CHO cells to obtain an efficient cell clone for production of human GCR on a large scale.

Supported by FAPESP, CNPq, Fundação Butantan

8.22 Optimization of the bioprocess for large-scale production of recombinant human thyroid stimulating hormone (hTSH) in a Chinese Hamster Ovary cell line, in spinner flasks

Sousa APB[1,3], Soares CRJ[2], Damiani R[2], Oliveira JE[2], Ribela MTCP[2], Bartolini P[2], Peroni CN[2], Tonso A[1,3], Pereira CA[1,3]

[1]Laboratório de Imunologia Viral, Instituto Butantan, SP, Brazil; [2]Instituto de Pesquisas Energéticas e Nucleares, CNEN, SP, Brazil; [3]Laboratório de Células Animais, Departamento de Engenharia Química, Escola Politécnica, Universidade de São Paulo, SP, Brazil

Introduction: Mammalian cell cultures are widely used for the production of recombinant and therapeutic proteins because of their ability to perform extensive post-translational modifications [(1)]. Among these mammalian cells, Chinese hamster ovary (CHO) cell lines that are dihydrofolate reductase deficient ($dhfr^-$) have been used extensively for this purpose (2). For the large-scale production of bioproducts such as vaccines and recombinant proteins, microcarriers (microspheres that serve as support for the cells) are being increasingly used. The use of microcarriers in stirred spinners flasks allows high cell densities and, consequently, high virus titers or recombinant protein expression. This technique was developed for the production of CHO-derived recombinant hTSH. **Objectives**: This study was mainly focused on the optimization of the large-scale production of a CHO cell culture in suspension; where the influence of several variables (agitation, ratio of inoculated cells to microcarrier mass, concentration ratio of substrates to metabolites such as glucose and lactate, and fetal bovine serum) on cell growth on Cytodex 1 microcarriers, was studied. **Methods**: CHO cells were grown α-MEM medium supplemented with 10% dialyzed fetal bovine serum, glucose (4.0 $g.L^{-1}$), $NaHCO_3$ (2.2 $g.L^{-1}$), gentamicin, penicillin/streptomycin, fungizone and methotrexate (100 nM), and in serum-free medium SFM-S-CHO. Cultures containing 100 mL cell suspension in spinner flasks (Bellco Company) were incubated at 37º C in 5% CO_2 and stirred at 40 rpm. Cytodex 1 microcarriers (GE Healthcare), $1g.L^{-1}$, were used. Viable cell number was determined using 0.1% crystal violet solution and was counted in a Neubauer chamber. Glucose and lactate were assayed using the YSI-2700 analyzer (Yellow Spring Instruments, USA). hTSH was determined via an in-house immunoradiometric assay (IRMA). **Results**: CHO cells cultured in serum-free medium, SFM-S-CHO, grew considerably. High cell viability was maintained during the cell culture resulting in a final cell density of 2.1- 2.5 x 10^6 cells/mL for 6 days in stationary cell culture phase, and an accumulated TSH concentration of 170 μg/500mL after 10 days cultivation **Discussion**: Since various factors affect mammalian cell growth on microcarriers, this work was carried out to determine the most appropriate conditions for obtaining high cell densities on Cytodex 1 microcarriers. The best experimental conditions, as well as the maximum cell densities obtained ($2.5x10^6$ cells/mL), were obtained when the following conditions were met: serum-free medium, agitation (40 rpm), and ratio of 25 cells/microcarrier. We conclude that agitation rate in spinner flasks, 10% FBS concentration used in the culture medium and the optimized ratio of inoculated cells to microcarriers exerted significant effects on CHO cell growth on Cytodex 1 microcarriers.

References: 1. Gilies *et al*., 1989; 2. Kaufman *et al*., 1985.
Acknowledgments: IPEN
Supported by FAPESP, CNPq, Fundação Butantan

8.23 Establishment of comet assay in *Biomphalaria glabrata* (Say, 1818)

Grazeffe VS[1], Tallarico LF[1], Garcia VSG[1], Schmidt BF[1], Okazaki K[2], Kawano T[1], Pereira CAB[3], Nakano E[1]

[1]Laboratório de Parasitologia, Instituto Butantan, SP, Brazil; [2]Centro de Biologia Molecular, Instituto de Pesquisas Energéticas e Nucleares, CNEN, SP, Brazil; [3]Departamento de Estatística, Instituto de Matemática e Estatística, Universidade de São Paulo, SP, Brazil

Introduction: The comet assay is a method developed to detect breaks in DNA. The fragments of damaged DNA show low molecular weight; on electrophoresis they migrate first in relation to the heavier fractions, acquiring the general appearance of a comet. This is a promising test for studies on genotoxicity, DNA repair, and environmental and human health monitoring. **Objective:** The aim of this study was to standardize the comet assay in *Biomphalaria glabrata*. **Methodology:** Hemolymph of *Biomphalaria glabrata* exposed to cyclophosphamide ($3.6.10^{-3}$ and $3.6.10^{-5}$ M) for10 days was mixed with low-melting point agarose and placed on slides prepared with normal melting point agarose. Cells were lysed overnight, and afterward exposed to an alkaline solution (pH>13) for 30 min. After electrophoresis, the slides were neutralized with Tris solution, stained with ethidium bromide and analyzed by fluorescence microscopy. The visual analysis was carried out by classifying comets in categories (0 to 3) according to the extension of the migration of the DNA. **Results:** The groups exposed showed increased migration of DNA when compared to the control group. **Discussion:** The data showed that a greater DNA damage was induced with the higher dose of cyclophosphamide. The results obtained demonstrate the sensitivity and capacity of this assay in detecting genotoxic effects induced by cyclophosphamide in *Biomphalaria glabrata*.

8.24 Histological and histochemical aspects of the accessory venom glands of juvenile and embryo *Crotalus durissus terrificus*

Giannotti KC[1], Sant'Anna SS[2], Fernandes W[2], Carneiro SM[1]
[1]Laboratório de Biologia Celular, [2]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: Venom glands of viperid snakes are composed of the main venom gland, the primary duct that connects this gland to the accessory gland, and the secondary duct that opens into the fang sheath. Histological studies on the accessory glands are very scarce. **Objectives**: The aim of the present study was to describe the histology and histochemistry of the accessory venom glands of the early embryo and juvenile *Crotalus durissus terrificus*. **Methods:** Venom glands from juvenile snakes were excised after decapitation of sedated snakes, fixed and prepared for histology; the embryos had their whole heads fixed and embedded in paraffin. HE and Mallory's trichrome stains, and histochemical reactions for detection of acid (Alcian blue) and neutral (PAS) mucopolysaccharides, and of proteins in general (bromophenol blue) were done in the histological sections. **Results:** In sagittal sections of the embryo heads, the accessory gland was seen as an anterior dilatation of the primordium of the primary duct that connects it with the main gland. Radiated epithelial cords are observed growing caudally, and a central dense epithelial core extends along the primary duct region. Tubular lumina are not yet formed. Abundant Alcian blue positive mesenchymal tissue is the substrate where the epithelial cords grow. These cords are not positive for neutral or acid mucopolysaccharides. In the juvenile snakes, the anterior region of the accessory gland shows secretory tubules formed mainly by large mucous cells, positive for both PAS and Alcian blue. The posterior region has elongated tubules formed by small cuboidal epithelial cells, Alcian blue and PAS negative. **Discussion:** In the early embryo, the secretory cells of both accessory and main venom glands of *C. d. terrificus* are not differentiated, and the tubules and gland lumina are not formed yet. In the juvenile glands, a mucous product from the accessory gland is added to the secreted venom of the main gland. The morphology of *C. d. terrificus* venom gland is similar to that of the *Vipera* species (1) of *Crotalus viridis oreganos* (2). Lubricant, protective or venom-activating properties of mucous secretion are discussed.

References: 1. Gans and Kochva Toxicon, 3: 61, 1965; 2. Mackessy *et al.* J. Morphol., 208: 109, 1991.

Supported by Fundação Butantan

8.25 Anaphylactic activity of murine IgG1 antibodies is independent of *N*-linked glycans and sialic acid residues

Silva SR[1], Casabuono A[2], Favoretto, BC[3], Jacysyn JF[1], Butera D[3], Moura da Silva AM[3], Macedo MS[1], Couto AS[2], Faquim-Mauro EL[3]

[1]Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, SP, Brazil; [2]Departamento de Química Orgânica, Universidade de Buenos Aires, Buenos Aires, Argentina; [3]Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil

Introduction: Antibodies are glycoproteins that play important role in pathogen/antigen elimination by the immune system. All antibodies contain branched sugar moiety attached at conserved positions in their Fc region, which is essential for maintaining the structure and the effector function of the antibodies, such as the interaction with cellular Fc receptors or the complement component C1q. However, it is not completely clear how differences in the *N*-linked oligosaccharide structure impact the biological activity of the antibodies. **Objective:** We investigated the influence of the *N*-linked oligosaccharide chains and the contribution of individual sugar residues with regard to the ability of the murine IgG1 antibody to elicit anaphylaxis. **Methods:** Two monoclonal IgG1 antibodies with the same antigenic specificity, one with *in vivo* anaphylactic activity and the other a non-anaphylactic IgG1, were used to characterize the *N*-linked oligosaccharide chains. **Results:** The two antibodies express the same amino acid sequence in the CH2 and CH3 domains of the heavy chain. Lectin-binding assays revealed different behaviors between the two IgG1 antibodies. Specifically, affinity chromatography analysis using *Triticus vulgaris* lectin (specific for sialic acid) showed that anaphylactic IgG1 binds preferentially. In addition, the released oligosaccharide chains of both antibodies were analyzed by high performance anion exchange chromatography using pulsed amperometric detection (HPAEC-PAD). The anaphylactic IgG1 displayed a complex profile due to monosialic and di-sialic acid containing oligosaccharides while non-anaphylactic IgG1 chromatogram was simpler. Moreover, mass spectrometry analysis by matrix assisted laser desorption ionization (MALDI-TOF) also showed a higher frequency of oligosaccharide chains containing fucose residues in anaphylactic IgG1 than in oligosaccharide chains obtained from non-anaphylactic IgG1. Although the enzymatic removal of fucose residues in anaphylactic IgG1 did not modify its biological activity, interestingly, removal of sialic acid residues from the carbohydrate chains of anaphylactic IgG1 resulted in total or partial decrease in the antibody/s ability to trigger mast cell degranulation and *in vivo* anaphylactic reaction. **Discussion:** The N-linked oligosaccharide chain and the sialic acid residues attached to the IgG1-Fc region are essential for IgG1 anaphylactic activity.

Supported by FAPESP, CNPq, CAPES

9. PIBIC program

9.01 Analysis of the fibrin(ogen)olytic activity of *Bothrops* protease A (BPA), a serine proteinase from the venom of *Bothrops jararaca*

Yamashiro ET[1], Prezoto BC[2], Bertholim L[1], Paes Leme AF[1], Camargo ACM[1], Serrano SMT[1]

[1]Laboratório Especial de Toxinologia Aplicada – CAT/CEPID, [2]Laboratório de Farmacologia, Instituto Butantan, SP, Brazil

Introduction*: Bothrops* protease A (BPA) is a serine proteinase isolated from *B. jararaca* venom. It is a heat-resistant, highly-glycosylated protein that migrates on SDS-PAGE as a single band of 67 kDa. Approximately 62% of the BPA molecular mass assessed by SDS-PAGE is due to carbohydrate moieties. Previous studies showed that BPA is able to prevent thrombus formation in rats caused by both stasis in the vena cava and endothelial injury in the jugular vein. In this study, we further characterized the proteolytic activity of BPA. **Objectives**: The aims of this study were: 1. to analyze the fibrin(ogen)olytic activity of BPA and the role of its carbohydrate moiety; 2. to analyze BPA enzymatic activity on plasma proteins; 3. to determine BPA enzymatic activity on peptide substrates. **Methods**: For hydrolysis experiments, BPA was incubated with proteins for various time intervals and at the following enzyme to substrate ratio: fibrinogen (1:500 and 1:1650); fibrin (1:500); and vitronectin, fibronectin and albumin (1:10). Hydrolysis was visualized by SDS-PAGE and Western blotting using an anti-fibrinogen antibody. Four different synthetic chromogenic substrates at different concentrations were incubated with BPA to analyze the hydrolysis kinetics. **Results and Discussion**: Here, we show that BPA is a potent fibrinogenolytic enzyme. After 5 min incubation with fibrinogen, BPA degraded alpha and beta chains, while the gamma chain was resistant to hydrolysis. Partial *N*-deglycosylation of BPA increased its hydrolytic activity on fibrinogen. With fibrin, BPA hydrolyzed the alpha chain, leaving the beta and gamma chains apparently untouched. Incubation of BPA with albumin, vitronectin and fibronectin for 1h at a 1:10 ratio caused only partial digestion of these proteins. BPA was also able to hydrolyze fibrinogen in plasma at a 1:1650 incubation ratio, as revealed by Western-blot analysis. The hydrolysis of chromogenic substrates showed that BPA is only active on substrates containing arginine at the P1 position. Taken together, these results suggest that BPA is a highly stable, specific fibrinogenolytic serine peptidase with potential therapeutic application.

Supported by CAT/CEPID-FAPESP, CNPq/PIBIC

9.02 Proteomic analysis of *Bothrops* venoms: comparative analysis of sub-proteomes

Kitano ES[1], Paes Leme AF[1], Ho PL[2], Furtado MF[3], Camargo ACM[1], Serrano SMT[1]

[1]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, [2]Centro de Biotecnologia, [3]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: Snake venoms are complex mixtures of components, which have a diverse mode of action both on prey and human victims. The knowledge of snake venom proteomic composition and its variability is important both in basic research and in the management of snakebites. **Objectives:** The aim of this study was to examine the venom complexity by comparison of venom from eight species of the genus *Bothrops* (*cotiara, insularis, moojeni, jararacussu, jararaca, bilineatus, neuwiedi, erythromelas*) in which the pharmacology, biochemistry, and examine the mechanism of action of various toxins. **Methods:** Venoms were examined by two-dimensional electrophoresis (2DE) to analyze similarities and differences. They were submitted to isoelectric focusing on pH 3-10 strips followed by electrophoresis on 12% SDS-polyacrylamide gels and Coomassie blue staining. Sub-proteomes of proteolytic enzymes were analyzed by 2D-immunostaining using specific antibodies and by 2D-gelatin zymography. Venoms were also analyzed by affinity chromatography. Samples were chromatographed in a heparin-Sepharose column, and proteins were eluted with increasing ammonium acetate concentration and analyzed by electrophoresis on 12% SDS-polyacrylamide gels. The proteins with high affinity for heparin were submitted to identification by in gel trypsin digestion and mass spectrometric analysis. Also, the pools of proteins from heparin-Sepharose chromatography were assayed for hemorrhagic and lethal activities in mice. **Results:** The visual inspection of the gels indicated notable differences between the observed 2DE profiles of these eight *Bothrops* species. All venoms had protein dispersed across the p*I* and molecular mass range of the gels. The venom gel images showed well-stained bands at molecular mass between 15 and 50 kDa and p*I* values ranging from 4 to 10. The *Bothrops* venoms also showed distinct subpopulations of metalloproteinases and serine proteinases in Western-blot and gelatin zymography analysis of 2DE of the venoms. Comparison of the proteins with affinity for heparin showed distinct heparinome profiles among the venoms. Proteins with high affinity for heparin were submitted to gelatin zymography and some of them displayed proteolytic activity. Protein identification by in gel trypsin digestion and mass spectrometric analysis revealed the presence of serine proteinases, metalloproteinases, phospholipases A2 and C-type like lectins among the proteins that bound with high affinity to heparin. *B. jararaca* venom hemorrhagic and lethal activities were concentrated in the pool of proteins that did not bind to heparin. **Discussion:** These approaches resulted in a more thorough understanding of the complexity of these venoms and provide insights to those who wish to focus on these venom subpopulations of proteins in future studies.

Supported by CNPq/PIBIC, CAT/CEPID-FAPESP

9.03 Screening of fractions with disintegrin activity from *Lachesis muta* venom

Agostino AC, Moura-da-Silva A, Della-Casa MS
Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil

Introduction: Disintegrins represent a family of cysteine-rich, low-molecular weight proteins occurring in venoms of various Viperidae snakes. They are nonenzymatic proteins that inhibit cell–cell interactions, cell–matrix interactions, and signal transduction, binding with a high affinity to numerous integrins. Disintegrins containing the Arg-Gly-Asp (RGD) motif bind to integrin receptors on the surface of both blood platelets (integrin $\alpha_{IIb}\beta_3$) and other cells. In search for new snake venom disintegrins, *Lachesis muta* venom was fractionated by HPLC. **Objectives:** The aim of this study was to isolate and to characterize disintegrins in the *L. muta* venom. **Methods:** A total of 150 μg venom of *L. muta* was fractionated in an HPLC system with a C18 reversed-phase column. All the fractions isolated by chromatography were characterized by SDS-PAGE. The biological activity was assessed by the capacity to inhibit ADP-induced human platelet aggregation. The fractions that showed 100% inhibition of ADP-induced human platelet aggregation were used in dose-response experiments. The immunological identity was also characterized with anti-disintegrin antibodies by Western blotting. **Results:** We isolated 22 fractions on an HPLC C18-reversed phase column. When these fractions were screened in protein excess (80μg/ml), only fractions 3 and 9 inhibited 100% ADP-induced platelet-aggregation. Further dose-response experiments of these two fractions showed that fraction 3 was the most potent inhibitor of platelet aggregation (IC_{50} ~ 9μg/mL). This fraction showed a single band when analyzed by SDS-PAGE, 14 kDa, which was also recognized by anti-disintegrin antibodies in Western blotting. This protein was named "lachesin." **Discussion**: Our results suggest that the disintegrin present in the *L muta* venom is represented by fraction 3, lachesin. This protein has the same elution time in the C-18-reversed phase column as other disintegrins characterized in other studies, as well as the same molecular weight, in SDS-PAGE, characteristic of all disintegrins. Anti-disintegrin antibodies also recognized this fraction. The lachesin showed high affinity with human platelet inhibiting 100% of platelet aggregation. These results suggest that lachesin interacts with $\alpha_{IIb}\beta_3$ present on platelets. Our study may contribute to research on new antagonists of integrins involved in pathologies dependent on cell adhesion.

Supported by CNPQ/PIBIC

9.04 Accidental scorpion stings seen at Hospital Vital Brazil: a three-decade comparative evaluation

Furlan E[1], França FOS[2]
[1]Escola de Enfermagem, Universidade de São Paulo, SP, Brazil; [2]Hospital Vital Brazil, Instituto Butantan, SP, Brazil

Introduction: Scorpion stings are common in tropical and subtropical regions. In Brazil, scorpion envenomations are frequent, mainly in Minas Gerais, São Paulo and Bahia. In 2005 35,809 cases were reported (16 cases/100,000 population) with 49 deaths (0.14%), but in children between 1-4 years the mortality was 1.15%. The most severe complications and deaths derive from systemic manifestations: cardiovascular, respiratory and rarely neurological abnormalities. The treatment of mild cases is symptomatic and antivenom is indicated only for moderate and severe cases. In 2005, more than 80% of cases were considered mild and did not receive serum therapy. Therefore, in some states, more than 90% of patients received antivenom and 27,503 antiscorpion vials were used. **Objectives**: To describe the epidemiological, clinical aspects and treatment of the accidents caused by scorpions attended at Vital Brazil Hospital. **Methods**: Scorpion stings that occurred in 1980 to 1984, 1990 to 1994 and 2000 to 2004 were analyzed from the clinical patient records. This abstract presents data from 1980-1984 and 2000-2004. Data were processed by Excel software. **Results**: A total of 1,413 scorpion stings were included, where 1982 showed the greatest number of cases (506) and in 2003 the least (84). The annual mean was 294.2 cases per year. Most of the cases occurred between October and January (Spring/Summer) when the annual temperature is higher. The causative species responsible for most of cases were *T. bahiensis*: 1341 (54.3%) and *T. serrulatus*: 177 (7.2%), and in 952 cases (38.5%) the scorpion was not identified. Accidents occurred mainly in the peripheral urban region, with poor sanitary conditions. Most of the patients were adults which numbered 1,978 (78.3%), and 547 (21.7%) were less than 13 years. A total of 1,464 (58.0%) were male and 1,061 (42.0%) female. Most of the stings occurred on the upper limbs (47.1%), which may be related to the activities of individuals at the time of the accident. Patients had shown signs of local and systemic envenomation. The local symptoms and signs were local pain: 2,332 (39.0%), hyperemia: 1,019 (17.0%) and edema: 1,479 (17.0%). Systemic abnormalities were uncommon: nausea and vomiting in 8 (0.1%), hypertension 331 (6.5%) and tachycardia 330 (6.2%). Some 15 cases (0.3%) did not have any symptoms and 154 (4.8%) presented with other alterations such as ecchymosis, dizziness, sudoresis and paresthesias at the site of the sting. Treatment with local infiltration (anesthesics) was given in 1,609 patients; 478 received analgesics, 170 hot local compress, 351 other kinds of treatments, and only 125 received serum therapy. **Discussion**: Our results indicate a decrease in the number of scorpion stings attended at the Vital Brazil Hospital, probably due to decentralization of health services with antiscorpion antivenom and to the training of personnel. Furthermore, there is a trend for a relative increase in accidents caused by *Tityus serrulatus*. Nevertheless, the majority of cases had good outcome without use of serum therapy. The selective use of different treatments could avoid the unnecessary use of antivenom. Analysis of data from 1990-1994 and the complete analysis of 3 periods of time are currently in progress.

Supported by CNPq/PIBIC, Butantan Institute
Acknowledgment to the staff of Vital Brazil Hospital

9.05 Serum therapy in snakebite: indications and determinations of early signs of poisoning

Kitazulo RC[1], França FOS[2]
[1]Escola de Enfermagem, Universidade de São Paulo, SP, Brazil; [2]Hospital Vital Brazil, Instituto Butantan, SP, Brazil

Introduction: Snakebites are a public health problem in tropical countries [(1)]. In 2005, 28,321 snakebites and 111 deaths were reported in Brazil, and in many regions more than 90% of patients bitten by snakes received antivenom. Serum therapy is a challenge mainly because of its inappropriate use: the unnecessary administration of the antivenom in patients bitten by nonvenomous snakes and in "dry bite" accidents, with economic implications and unnecessary exposure of patients to antivenom. Also, when the antivenom is indicated, it is not always given early enough and correctly. **Objectives:** The aims of this study were: to evaluate serum therapy in patients bitten by snakes, seen at Vital Brazil Hospital from 2000 to 2004; to identify the reasons and the frequency of using (or not using) serum therapy; and to verify the clinical and laboratory alterations observed in patients bitten that justified the use of the antivenom. **Methods:** Data from 769 patient records were collected and analyzed by Epi-Info. **Results:** Serum therapy was administered to 409 of 769 patients. The clinical and laboratory alterations were: edema in 329 (80.4%) and ecchymosis in 265 (64.8%); the clotting time was altered in 271 (66.2%). When the snakes were brought to HVB, they were immediately identified to see if the patient had been envenomed by it: 235 (71.21%) were and 95 (28.79%) were not. From the total of patients that brought the poisonous snake, 188 (80%) received serum therapy, 29 only presented with swelling, 2 only presented with altered clotting time, 23 presented with swelling and altered clotting time, 23 showed ecchymosis and edema (2 alterations), 52 had swelling, altered clotting time and ecchymosis (3 alterations) and 1 showed visual changes. This last case was diagnosed as *Crotalus* envenomation. The number of patients who did not receive antivenom when the snake was identified as poisonous was 47 (20%): 34 because of "dry bite," two by a presumed accident with bothropic venom in the ocular globe, 10 patients had already received bothrops antivenom in another healthcare center and one by occasional ingestion of poison while handling the snake. For those who had not brought the causative agent, 221 (50.34%) patients received serum therapy: 18 presented with only swelling, 24 showed swelling and altered clotting time, 36 had ecchymosis and swelling (two alterations), 66 showed swelling, altered clotting time and ecchymosis (three alterations), and two displayed only visual alterations. This result demonstrates that swelling, ecchymosis and altered clotting time are common and precocious alterations in *Bothrops* accidents. **Conclusion:** There is a need to improve the criteria for antivenom therapy in our country: pointing out to healthcare professionals the main and early alterations that indicate envenomation and also that a significant number of accidents do not need antivenom ("dry bites" and accidents caused by nonpoisonous snakes). This study suggests an excessive use of anti-snake serum therapy in Brazil when examining the data from this research.

1-References: WHO, Progress in the characterization of venons and standardization of antivenoms. Geneva, 1981
Acknowledgment to Vital Brazil Hospital and Instituto Butantan staff.
Supported by CNPq/PIBIC

9.06 Profile of antimicrobial susceptibility of bacteria strains isolated from secondary infection following *Bothrops jararaca* bites

Yassui ASA[1], Franzolin MR[1], Fan HW[2], Malaque CMS[2], Grego KF[3], Calleffo[3] MEV, Sant'Anna OA[4]

[1]Laboratório de Bacteriologia, [2]Hospital Vital Brazil, [3]Laboratório de Herpetologia, [4]Laboratório de Imunoquímica, Instituto Butantan, SP, Brazil

Introduction: Necrosis induced by *Bothrops* envenomation can facilitate secondary infection and lead to abscess formation at the site of the bite. In these cases, adequate treatment requires prompt surgical drainage combined with administration of appropriate antibiotics. Snakes have in their oral cavity a variety of bacteria derived from the varied feeding and way of life, which can be potential pathogens in the infections observed accompanying snakebites. The aerobic Gram-negative bacilli (mainly *Morganella morganii* and *Providencia sp.*) were the bacteria more frequently found in infections after *Bothrops jararaca* envenomation, being similar to those encountered in the oral cavity of the snakes, representing the source of pathogens. **Objectives:** The aim of this study was to evaluate the antibiotic susceptibility rates of bacteria strains isolated from abscesses of 23 patients attended at the Vital Brazil Hospital and from the oral cavity of 65 *Bothrops jararaca* snakes captured in the metropolitan area of São Paulo. **Methods:** The bacteria were tested by the Kirby-Bauer method for antimicrobial susceptibility, utilizing commercially available sensitivity discs and Muller-Hinton agar. **Results:** All bacteria tested were aerobic Gram-negative bacilli and were 100% sensitive to amikacin, ceftazidime, ceftriaxone, chloramphenicol, trimethoprim + sulfamethoxazole and gentamicin. Resistance was observed for cephalotin (65%), ampicillin (35%), amoxicillin/clavulanic acid (17%) and tetracycline (17%). The samples of bacterial strains from snakes showed resistance to cephalotin (50%), ampicillin (32%) and tetracycline (15%). Multiresistant strains were not found. **Discussion:** These results show a variable antimicrobial susceptibility of aerobic Gram-negative bacilli, which was similar in the two groups studied. The profile of antimicrobial sensitivity of isolated bacterial strains showed a lack of antibiotic selection pressure, compatible to community infection. In order to choose the best antimicrobial therapy, it is important to know the predominant bacteria involved with infection and its sensitivity to antibiotics.

Supported by FAPESP, CNPq/PIBIC

9.07 Effects of toxins isolated from *Micrurus lemniscatus* snake venom on muscarinic acetylcholine receptors in rat hippocampus

Medeiros WAA[1], Casais e Silva LL[3], Lebrun I[2], Abdalla FMF[1], Sandoval MRL[1]
[1]Laboratório de Farmacologia, [2]Laboratório de Bioquímica e Biofísica, Instituto Butantan, SP, Brazil; [3]Laboratório de Venenos Animais, Universidade Federal da Bahia, BA, Brazil

Introduction: Toxins isolated from venoms of Old World Elapids have been extensively studied as sources of presynaptic PLA2s and postsynaptic neurotoxins. These toxins have been shown to be valuable tools for the characterization of the structure and function of muscular cholinergic receptors as well as to identify subtypes of nicotinic and muscarinic receptors that control specific functions in the brain. Therefore, few investigations have dealt with isolated toxins from venoms of Brazilian snakes of the genus *Micrurus* (coral snakes, family Elapidae) and their effects on the brain. There is a lack of information for *M. lemniscatus* with respect to toxins from *Micrurus* snakes on cholinergic neurotransmission, particularly the muscarinic acetylcholine receptors. Therfore, the study of the effect of these toxins on the latter receptors would be interesting. **Objectives:** The present study was designed to investigate the effect of different purified peaks obtained from venom of the *Micrurus lemniscatus* snake on muscarinic acetylcholine receptors in the hippocampus of male rat. **Methods:** Different purified peaks obtained from venom of the *M. lemniscatus* snake (P01, P02, P03, P04 and P05) were isolated by RP-HPLC and identified for spectrophotometer mass type MALDI-TOF. For binding assays, hippocampal membranes were obtained from adult male rats as described [(1)]. In saturation experiments, hippocampal membranes were incubated with [^{3}H] quinuclidinyl benzylate ([^{3}H]QNB) (0.05 - 8.0 nM) in the absence and presence of atropine (1 μM) (non-selective muscarinic antagonist). In competition experiments, hippocampal membranes were incubated with [^{3}H]QNB (concentration near the k_d value), in the absence and presence of increasing concentrations of atropine (positive control) or different purified peaks (30^{o}C/1h). **Results:** Scatchard plot analysis of specific binding yielded a dissociation constant (K_D)=0.88 ± 0.13 nM and binding capacity (B_{max})=1459.40 ± 235.26 fmol/mg protein (n=5) in hippocampal membranes. In competition assays, the pK_i value was 8.96 ± 0.08 (n=4) for atropine. P01, P02, P03 and P04 peaks purified from *M. leminscatus* venom, were not able to reverse the specific binding of [^{3}H]QNB. On the other hand, P05 peak was able to displace [^{3}H]QNB in hippocampal membranes from rats. **Discussion**: The results indicate that the venom of the *Micrurus lemniscatus* snakes contains at least one toxin with activity on muscarinic acetylcholine receptors in the hippocampus of male rats.

References: 1. Neuroendocrinol. 80: 379, 2004
Supported by FAPESP, CNPQ/PIBIC

9.08 *Bothrops* snake venoms induce cleavage of C1INH allowing classical complement activation

Alves Pessoa LR[1], Queiroz-Pidde G[1], Farias C[1], Spadafora-Ferreira M[1], van den Berg C[3], Furtado MF[2], Tambourgi DV[1]

[1]Laboratório de Imunoquímica, [2]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil; [3]Departamento de Farmacologia, Terapêutica e Toxinologia, University of Wales, College of Medicine, Cardiff, UK

Introduction: Envenomations by pit vipers (family Viperidae, subfamily Crotalinae) constitute a public health problem in many regions of the world. In Brazil, most snakebite envenomations are inflicted by a large and widely distributed group of snakes from this subfamily in the genus *Bothrops* (23 species in Brazil*)*. Their venoms are composed of a great number of toxins, which are capable of acting on tissue and plasma components with consequent toxic and pharmacological effects. Clinically, patients bitten by *Bothrops* usually show edema, systemic bleeding, thrombocytopenia and prolongation of whole blood clotting time. These symptoms result from the 3 main activities of bothropic venom: proteolytic, with local inflammatory edema at the snakebite site; hemorrhagic, with endothelium damage and systemic bleeding; and procoagulant, leading to the consumption of clotting factors and disrupting the equilibrium of blood coagulation. **Objectives:** In the present study, we investigated the action of venoms from 19 species of snakes from the genus *Bothrops* occurring in Brazil, on the complement system in *in vitro* studies. **Results and Discussion**: All venoms studied were able to activate the classical complement pathway in the absence of antibody in a dose dependent manner, as evaluated in hemolytic assays. This activation was caused by the cleavage of C1Inh by proteases abundantly present in these venoms, which disrupts complement activation control. Generation of C3a, C4a and C5a were also detected in sera treated with some of the venoms. Since C1Inh plays important roles in both the regulation of classical complement activation and the regulation of coagulation, these results suggest that dysregulation of complement activation and coagulation, mediated by Bothrops protease induced C1Inh cleavage, contributes to the inflammatory pathogenesis of Bothrops envenomation.

Supported by FAPESP, CNPq/PIBIC

9.09 Crotoxin isolated from *Crotalus durissus terrificus* down-modulates T cells and antibody responses induced by human serum albumin in mice

Ricardi R, Favoretto BC, Silva SR, Takehara HA, Faquim-Mauro EL
Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil

Introduction: The immune system is strongly efficient in eliminating distinct pathogens/antigens by effector mechanisms mediated by cells or antibodies. However, some pathogens or their products are able to exert a suppressive effect on the production or activation of immune responses. It was demonstrated that crotalic venoms have the ability to down-modulate the immune system. We have verified that the crotoxin (ctx) isolated from *Crotalus durissus terrificus* venom (Cdt) suppresses the antibody production induced by human serum albumin (HSA), when injected 1 h before or 24 h after the immunization of BALB/c mice with HSA in $Al(OH)_3$ gel or complete Freund's adjuvant (CFA). **Objectives:** We evaluated the ability of T lymphocytes obtained from HSA-immunized mice and treated with ctx to proliferate upon *in vitro* stimulation. The ability of ctx to modulate the anti-HSA antibody response was also studied. Furthermore, the suppressive effect of the ctx on the antibody production was investigated in a different mouse strain (C57BL/6). **Methods:** Cell suspensions were prepared from spleens or lymph nodes from BALB/c mice immunized 7 days before with HSA (100 μg/animal) adsorbed in $Al(OH)_3$ gel (1 mg/animal) or emulsified with CFA, s.c., and injected or not with ctx (5 μg) s.c. 1h before or 24h after immunization. The cells were *in vitro* stimulated with HSA, ctx, ConA or anti-CD3 mAb for 48 h, and T cell proliferation was measured by the MTT assay. The effect of ctx on anti-HSA antibody production was evaluated in BALB/c mice s.c. immunized with HSA (100 μg/animal) in $Al(OH)_3$ gel or in CFA, and after 3 or 14 days received s.c. the ctx (5 μg/animal) in PBS. Another group of mice was only immunized with HSA or received ctx 1 h before the HSA-immunization. Groups of C57BL/6 mice were immunized s.c. with HSA in $Al(OH)_3$ gel or in CFA and received the ctx 1h before or 24 h after the immunization. After 14 days, the mice were bled, received the HSA-booster and bled after an additional 7 days. **Results:** Lower proliferative responses were observed for T lymphocytes from HSA-immunized mice treated with ctx when compared to those obtained from HSA-immunized mice. The ELISA results showed that ctx was able to down-regulate the anti-HSA IgG1 and IgG2a antibody production when administered 3 days after the immunization but not 1 h before the antigenic booster (14 day after immunization). The suppressive effect of ctx on anti-HSA antibody production was also observed in C57BL/6 mice. **Discussion:** Our results showed that ctx exerted a suppressive effect not only on antibody production but also on the ability of T cells to respond to antigen and mitogen stimulation. In addition, the ctx was effective in down-modulating the generation of immune response induced by the first immunization with the antigen, but not the activation of the memory response elicited by the antigenic booster.

Supported by CNPq/ PIBIC

9.10 Investigation on the direct injection of the toxin Tx2-6 (eretin) on the paraventricular hypothalamic nucleus and induction of penile erection

Ravelli KG[1], Magalhães L[1], Magnoli FC[2], Troncone LRP[1]
[1]Laboratório de Farmacologia, [2]Laboratório de Imunoquímica, Instituto Butantan, SP, Brazil

Introduction: Twenty-five percent of the male population over 65 years complain of some degree of erectile dysfunction. Penile erection is the result of a complex neuro-vascular physiological process that induces smooth muscle relaxation and blood flow to the cavernous bodies. Such neuronal control has both a central and peripheral origin. The Brazilian spider Phoneutria nigriventer is very aggressive and causes thousands of human accidents every year. Patients show a strong local pain, sweating, salivation and tremors and in rare cases a persistent penile erection (priapism) is observed. The crude venom of this spider has been fractionated and the components responsible for the priapism were identified and called eretins. These toxins were sequenced and called Tx2-5 and Tx2-6. In our previous study, mice treated intra-peritoneally with appropriate doses of one of the eretins expressed the typical priapism, and their brains demonstrated an increased expression of the early gene c-fos, a neuronal marker of hyper activation. Among the few brain nuclei activated, one called our attention since it has been implicated in central modulation of penile erection: the paraventricular nucleus of the hypothalamus (PVN). **Objectives:** This study addressed the important issue of whether the direct application of eretin to the PVN could induce penile erection, implicating the central effect in this action. **Methods:** Twenty male Swiss mice were surgically implanted with an intracerebral guide cannula directed at the PVN. Two days after surgery mice were injected in the PVN with decreasing amounts of toxin, ranging from 3 μg to 0.0006 μg dissolved in a fixed volume of 3 μL. Signs of toxicity were described by two trained observers, especially the occurrence of penile erections. **Results:** Mice presented convulsions and death with the higher doses while the lower doses induced tremors, salivation, respiratory distress and piloerection but no penile erection. **Discussion:** Therefore, according to these results eretin-induced priapism cannot be attributed to a central effect of the toxin. In a parallel study performed in our laboratory, the intra-cavernous injection of small amounts of eretin can induce priapism, arguing against a central effect. The observed activation of PVN described earlier could be attributed to a non-specific activation produced by the stress of intoxication since PVN is well known as a stress response relay.

Supported by CNPq/PIBIC, Fundação Butantan

9.11 Effects of jararhagin, a toxin from *Bothrops jararaca* venom, on the expression of adhesion molecules in human melanoma cells

Capitanio JS, Klein A, Ruiz IRG
Laboratório de Genética, Instituto Butantan, SP, Brazil

Introduction: Malignant melanoma is the most severe skin cancer, originating from the transformation of melanocytes, with high metastatic risk. Several changes in the adhesive profile of these cells take place during the malignant process. For instance, the switch from the E-cadherin to N-cadherin expression signals the beginning of the vertical growth phase of the tumor. The expression of integrins is also important, allowing the adhesion of malignant cells to blood vessels and to new tissues, also favoring vertical growth and metastasis. The toxin jararhagin, from *B. jararaca* venom, has several protein domains, among them the disintegrin domain which binds to tumor cell integrins and may inhibit metastasis. **Objectives:** The aim was to analyze the effects of jararhagin on the expression of integrin and cadherin subunits in malignant melanoma cell lines and evaluate possible alterations this toxin may cause to repetitive DNA sequences. **Methods:** SK Mel-28 and Mel-85 cell lines, established from human malignant melanoma metastasis, were treated with 30 and 45 ng/μL jararhagin, for 24 h. Total RNA obtained with the tryzol reagent was used for cDNA reverse transcription. The cDNA pool was template in PCR reactions with individual primer pairs for integrins α_v, α2, α3, α5, α6, β1, β3, β4, β5, β6 and classical E, P, N cadherins. Total DNA was extracted with the current phenol/chloroform protocol. The RAPD technique was used to access the genome integrity and stability with OPA (1, 2, 3, 4, 5, 8, 13, 14, 17, 20) and OPB (1, 2, 5, 6, 7, 8, 9, 11, 19, 20) primers. The PCR results were visualized in agarose gels. **Results:** The observed changes induced by jararagin on the expression of adhesion molecules were cell-type specific. E-cadherin was up-regulated and α5 integrin was down-regulated in both melanoma cell strains. Jararhagin up-regulated the expression of integrin β6, cadherins E and N in SKMel-28 cells, and of integrins αv, α3 and E-cadherin in Mel-85 cells. Integrins αv, α5, α6, β1 and β3 were down-regulated in SKMel-28; α5 and β5 were down-regulated in Mel-85. SKMel-28 did not express integrins α2 and α3; Mel-85 did not express integrins α2, α6, P and N cadherins. As to the effects of jararagin on DNA, the RAPD patterns showed an increased number of bands in 30 % of SKMel-28 samples. No alterations were visualized in Mel-85 cells. **Discussion:** Jararhagin seems to be an appropriate anti-melanoma agent, considering the expression changes induced in SKMel-28: the down regulation of integrins subunits α6, α5, αv, β3 and β1 may inhibit the MAPK signaling pathway. Up regulation of αvβ3 inhibits tumor suppressors, while α6β1 and α5β1 promote expression of MMP-2. Therefore, the down regulation observed may promote an anti-tumor effect. The Mel-85 results showed increase in αv, and decrease of α5 and β5 expression. The increase in E-cadherin expression, with jararagin treatment in both cell lines, suggests that keratinocytes would regain control over tumor cells and inhibit their detachment from the epidermis *in vivo*. Possible changes in the expression of other molecules downstream to the cadherin and integrin signaling pathways will be studied in melanoma cell lines. Cloning and sequencing of the main altered bands in the RAPD profiles will also be performed.

Supported by CNPq/PIBIC, FAPESP

9.12 Evaluation of the presence of the human papillomavirus in patients submitted to gynecological evaluations that were chronic consumers or not of the *P. aquilinum* bracken fern

Encarnação TBC[1], Silva IDCG[2], Lima AA[1,3], Pitol BCV[3], Timóteo MA[3], Moran MS[3], Miranda PM[3], Felix PM[1], Giovanni DNS[1], Campos SRC[1], Beçak W[1,4], Stocco dos Santos RC[1]

[1]Laboratório de Genética, Instituto Butantan, São Paulo, SP, Brazil; [2]Laboratório de Tocoginecologia, Universidade Federal de São Paulo, SP, Brazil; [3]Escola de Farmácia, Universidade Federal de Ouro Preto, MG, Brazil; [4]Departamento de Genética, Universidade Federal de Pernambuco, PE, Brazil

Introduction: Cervical cancer is the second most important cause of death in women, after breast cancer. It is known that the presence of the human papillomavirus (HPV) leads to the formation of warts and eventually the possible development of cervical cancer. The papillomaviruses are non-enveloped viruses with an icosahedric capsid composed of 72 subunits and circular double-stranded DNA of approximately 8 kbp. There are several co-factors that increase the risk of cervical cancer. Among these is the consumption of the bracken fern *Pteridium aquilinum*, which possesses mutagenic substances, such as quercetin and ptaquiloside. It has been observed that these substances cause chromosomal alterations. **Objective:** The aim the present study was the investigation of the correlation between the consumption of *P. aquilinum* bracken fern and the presence of HPV and the detection of similar viral DNA sequences in peripheral blood and cervical samples. **Methods:** For the study, 70 women in reproductive age were selected, which were chronic consumers or not of bracken fern, showing different levels of cervical lesions or no symptom at all. Their peripheral blood was also collected for lymphocyte culture and for molecular studies. Samples of cervical material were obtained from the same patients for molecular studies. The extent of chromosomal aberrations was established in cultured lymphocytes and the presence of viral DNA was evaluated by PCR using generic oligonucleotide primer pairs (MY09/11) and subsequent enzymatic digestion for typing. A group of 25 patients was selected as control: the same procedures were carried out for the same analysis, their clinical conditions were evaluated, and they were selected from a different region, where there is no exposure to the co-factor. **Results and discussion:** the results indicated: 1- the influence of habits: the 70 patients shared similar social-religious behavior and feeding habits, except the co-factor consumption, with two different groups, exposed or not to the co-factor; 2- the relationship between the infection in the cervix and the virus sequences in the blood: the presence of virus DNA was only verified in patients with HPV detected in cervical samples, 3- the clastogenic viral action detected in lymphocytes of patients with different levels of cervical lesions; 4. the signicant differences detected comparing the levels of chromosomal aberrations demonstrated in lymphocytes in patients exposed or not to co-factor, 5- the significant differences detected comparing the levels of chromosomal aberrations determined in lymphocytes in patients presenting with HPV or not; and 6- the significant differences detected comparing the levels of chromosomal aberrations determined in lymphocytes in patients presenting with HPV exposed to co-factor or not. The data obtained allow relevant discussion about the interaction virus-co-factor–host chromatin: obviously there is a distinct effect in the host-chromatin of the virus and the co-factor, which can also be verified as synergistic or not.

Supported by PIBIC/CNPq, FAPESP, FACEPE, CNPq

9.13 Bovine papillomatosis in dairy herd: preliminary evaluation in Minas Gerais, Brazil

Miranda PM[1,2], Pitol BCV[1], Moran MS[1], Timóteo MA[1], Trindade C[1], Giovanni DNS[1], Campos SRC[1], Encarnação TBC[1], Félix PM[1], Ferraz OP[1], Assaf SLMR[1], Beçak W[1,3], Stocco dos Santos RC[1], Lima AA[1,2]

[1]Laboratório de Genética, Instituto Butantan, SP, Brazil; [2]Escola de Farmácia, Universidade Federal de Ouro Preto, MG, Brazil; [3]Departamento de Genética, Universidade Federal de Pernambuco, PE, Brazil

Introduction: Bovine papillomaviruses (BPV) associated with co-factors may result in warts and tumors of the alimentary and urinary tracts in cattle. These viruses have a worldwide distribution; in Brazil, affected animals are mainly found in dairy farms. Information on epidemiological aspects of the bovine papillomatosis is scarce. Detection of these viruses in lymphocytes of peripheral blood and other body fluids such as milk, collostrum, and urine shows different forms of horizontal and vertical transmission. Furthermore, the detection of the virus in seminal fluid, frozen semen and uterine flushing demonstrates that the virus can interfere with the reproductive status of the animals. The co-factor *Pteridium aquilinum* has been described as acting in the development of cancer related to BPV infection. The first step in finding a way to control the endemic situation of the BPV infection in our country is to make a detailed report about its prevalence and distribution. **Objectives:** In Ouro Preto, Minas Gerais, Brazil, we could detect the first dairy herds with severe cutaneous lesions linked to BPV, and our aim was to investigate the type of lesions, the presence of specific BPV types, the presence of virus genome in the lesions and peripheral blood, and to determine the presence of chromosome aberrations in lymphocytes. **Methods**: Lesion fragments and peripheral blood samples were collected from affected bovines. Morphological alterations in infected tissues have been analyzed through anatomopathologic studies performed on wart fragments. All the samples were investigated for the presence of papillomavirus genome sequences by PCR techniques, and confirmed as being infected by papillomavirus. Blood samples were incubated in RPMI 1640 medium, supplemented with 20% fetal serum and 2% phytohemagglutinin, and kept for 72 hours at 37°C. Colchicine (16ug/ml) was added for 1 h; the material was centrifuged and hypotonization (KCl 0.075 M) and fixation (methanol: acetic acid 3:1) were performed. The slides were stained with 3% Giemsa in phosphate buffer, pH 6.8, and analyzed with a light microscope. **Results and Discussion:** The anatomopathologic studies showed different severities of lesions, harboring different virus types. The presence of chromosome markers was detected in the cytogenetic evaluations. An important point is the presence of the co-factor *Pteridium aquilinum* in the pastures of the region. In this region, this plant also included in the human diet, with not completely evaluated consequences. Thus, the high frequency of papillomavirus associated with a huge presence of co-factor emphasizes the need for effective control.

Supported by CNPq/PIBIC, FAPESP

9.14 Adhesion pattern of different atypical enteropathogenic *Escherichia coli* serotypes in Caco-2 and T84 lineages cells

Barbosa CFM, Soares ES, Zarpelão E, Ruiz RC
Laboratório de Bacteriologia, Instituto Butantan, SP, Brazil

Introduction: The EPEC category has been associated with diarrhea in several countries, including Brazil. The virulence factors of the typical EPEC are encoded both in the LEE region and in the EAF plasmid, while the atypical EPEC does not carry the EAF plasmid, having the virulence factors encoded only in the LEE region. Atypical EPEC produces intimin and secretes Tir and Esp proteins. However, the mechanisms that regulate this production as well as the alterations caused by atypical EPEC infection in this host-pathogen interaction are still unknown. As an extracellular bacterium, its pathogenicity is related to cell adhesion. The typical EPEC always displays the localized adhesion pattern (LA), while the atypical may show additional patterns: indefinite (IND), localized like (LAL), aggregative (AA), diffuse (DA) or non-adhesive (ND). The fact that the adhesion patterns were described using HEp-2 larynx derived cells may not reflect an *in vivo* infection. Although the importance of atypical EPEC has been increasing as a cause of diarrhea, very few studies focusing on this microorganism have been reported. **Objectives:** The aim of the present study was to characterize the adhesion phenotype of several atypical EPEC serotypes interacting with different polarized cell lines derived from human intestinal epithelium. **Methods:** Adhesion assays were performed with 18 atypical EPEC samples (4 LA; 5 IND; 6 LAL; 2 AA; 1 NA) in Caco-2 and T84 polarized cells, at 3, 6 and 9 h, in the presence or absence of mannose. HEp-2 cells were used as control. **Results**: No differences were observed in the adhesion pattern of the atypical EPEC samples, previously determined in HEp-2, with both Caco-2 and T84 lines. Nonetheless, some samples, previously IND at 6 h, demonstrated a pattern at 9 h, mostly LAL. The absence of mannose made no difference. **Discussion**: The fact that the adhesion pattern is the same in cells of different origins suggests that this process is preserved either through the use of conserved receptors or through distinct receptors. Thus, the fact that the ND sample does not adhere to the intestinal cells is surprising. Interestingly, in the absence of mannose, all phenotypes were unchanged, suggesting that type I fimbriae are not involved in the process.

Supported by FAPESP, FUNDAP, CNPq/PIBIC

9.15 Development of a conjugated vaccine against pathogenic *E. coli*

Andrade GR[1], De Paula DB[1], New RRC[2], Barbaro K[3], Ozaki CY[1], Dos Santos MF[1], Sant'Anna OA[4], Domingos MO[1]

[1]Laboratório de Bacteriologia, [3]Laboratório de Imunopatologia, [4]Laboratório de Imunoquímica, Instituto Butantan, SP, Brazil; [2]Proxima Concepts, Londres, UK

Introduction: The serogroup O111 of *E. coli* can be found in three different categories of pathogenic *E. coli*, namely enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC). In addition, the O111 serogroup is the mainly responsible for child diarrhea in endemic areas of Brazil and outbreaks of bloody diarrhea in developed countries. Previous results obtained in our Laboratory demonstrated that the O111 polysaccharide is an excellent candidate for use as an antigen in the formulation of a conjugated vaccine against three different categories of diarrheagenic *E. coli*. **Objectives:** The aim of this study was to conjugate the polysaccharide O111 to a protein in such a way that when used as a vaccine formulation it would be able to raise antibodies capable of recognizing all three diarrheagenic categories of live *E. coli* and inhibit their adhesion to epithelial cells. **Methods**: Detoxified O111 polysaccharide was conjugated either to cytochrome C or BSA proteins by using adipic acid dihydrazide as cross-linker. The conjugate was incorporated either in silica, alum or emulsified in the formulation used by Butantan Institute for human vaccines. Mice and rabbits were immunized with the conjugate formulations and the antibody titer was determined by ELISA. Flocculation was employed to assay for recognition of live *E. coli* bacteria and inhibition of adhesion to human epithelial cells was determined *in vitro* by light microscopy. All the results obtained with the conjugated vaccines were compared to the results obtained with antibodies raised in rabbits immunized with either whole bacteria or LPS. **Results:** Antibodies from mice and rabbits immunized with the conjugated vaccines were able to recognize and aggregate samples of all three categories of live O111 bacteria, including clones derived from cat and dog. These antibodies were also able to inhibit completely the adhesion of O111 *E. coli* of all three categories to human epithelial cells *in vitro*, but do not interfere with adhesion of any other serogroup. Furthermore, biofilm formation on plastic and glass surfaces was completely inhibited by these antibodies. **Discussion**: O111 detoxified polysaccharide conjugated to proteins by ADH methods, and incorporated into a vaccine formulation already approved for human use, is able to induce antibodies that recognize and neutralize the adhesion of live bacteria belonging to EHEC, EPEC and EAEC categories.

Supported by CNPQ/PIBIC

9.16 Commensal *E. coli* influences cellular responses to typical and atypical EPEC strains

Paiva FPT, Ozaki CY, Domingos MO, Sircili MP
Laboratório de Bacteriologia, Instituto Butantan, SP, Brazil

Introduction: Quorum sensing is used to designate a mechanism of gene regulation depending on cellular density. The bacteria produce substances that accumulate in growth media, and when these substances reach a threshold concentration, a variety of responses can occur. These molecules are called autoinducers, and the phenomenon is called quorum sensing. Quorum sensing is widespread in gram-negative and gram-positive bacteria and is involved in various cellular mechanisms, including virulence regulation. At least, two quorum-sensing systems were described in literature and were designated LuxI/LuxR AI-1 (autoinducer 1) system and LuxS/AI-2 (autoinducer 2) system. In enteropathogenic *Escherichia coli* (EPEC), and enterohemorrhagic *Escherichia coli* (EHEC), type III secretion system is quorum-sensing regulated (LuxS/AI-2 system). **Objectives:** This study was developed to determine the possibility of crosstalk among EPEC and other enterobacteria, in intestinal infections. **Methods**: Adherence assays: Bacterial strains were grown for 18 h in LB medium at 37°C. From the non induced overnight cultures, 10^5 CFU (equivalent to an OD600 of 0.05) was added to HeLa cells, incubated for 3 h at 37°C with 5% CO2, washed with PBS, fixed with methanol, and stained with Giemsa stain. For quantification of adherence, after a 3-h incubation, the nonadherent bacteria were removed by washing with PBS and the HeLa cells were lysed with 1% Triton. Serial dilutions of the bacterial cells were plated on LB agar plates, and CFU were counted. **Results:** Apparently, the inoculation of cell cultures with commensal *E. coli* influences the cellular responses to typical and atypical EPEC strains. **Discussion:** Using commensal *E. coli* in adherence assays, we observed a different interaction of typical and atypical EPEC with HEp 2 cells.

Supported by CNPq/PIBIC

9.17 Detection of virulence genes in atypical EPEC strains

Santos TO, Porangaba TM, Franzolin MR, Sircili MP
Laboratório de Bacteriologia, Instituto Butantan, SP, Brazil

Introduction: Diarrhea continues to be one of the most common causes of morbidity and mortality among infants and children, especially in developing countries. Among the bacterial pathogens, diarrheagenic *Escherichia coli* (DEC) is an important agent of endemic and epidemic diarrhea worldwide. The diarrheagenic *E. coli* strains can be classified into six main pathotypes, based on specific virulence properties, clinical features, association with serotypes O:H, epidemiological aspects, and patterns of interaction with cell strains. Enteropathogenic *Escherichia coli* (EPEC) cause a histopathological lesion known as "attaching and effacing" (A/E). Typical EPEC differs from atypical EPEC by the presence of a plasmid called EPEC adherence factor (EAF) that encodes the bundle-forming pilus (BFP). Atypical EPEC comprises a very heterogeneous group. We developed multiplex PCR reactions in order to identify virulence genes present in other DEC pathotypes in an atypical EPEC collection of 89 samples. **Objectives:** The aim of the study was to search for virulence genes of DEC in atypical EPEC. **Methods:** DNA templates for PCR were obtained from overnight *E. coli* cultures that were pelleted, resuspended in 500µl of sterile deionized water and boiled for 10 min. The multiplex PCR was developed by combining specific primer pairs. Each multiplex reaction was performed in a 50-µl final volume containing 1µl of the template DNA, 0.2mM dNTPs, 10mM Tris-HCl (pH8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 2 U *Taq* DNA polymerase (Invitrogen) and 10 pmol of each primer (Biosynthesis). An initial denaturation step was carried out at 94°C for 5 min, followed by thirty-five cycles of amplification (denaturation 45 sec at 94°C, annealing 1 min at 62°C and extension 2 min and 15 sec at 72°C) and a final extension step at 72°C for 9 min. Amplified samples were detected by 1% agarose gel electrophoresis in Tris-borate-EDTA buffer and ethidium bromide staining. **Results:** More than 50% of the strains studied showed positive results for at least one of the genes studied. The *astA* gene was the most detected and *hly* gene showed the lowest frequency among the strains studied. **Discussion:** Based on the results, we can say, as expected, that some strains of atypical EPEC strains carry virulence genes common to other DEC.

Supported by FAPESP, CNPQ/PIBIC

9.18 Comparative proteomic analysis of the outer membrane and periplasmic fraction between typical and atypical enteropathogenic *Escherichia coli*

Taddei CR[1], Oliveira FF[1], Piazza RM[1], Paes Leme AF[2], Serrano SMT[2], Sant'Anna OA[3]

[1]Laboratório de Bacteriologia, [2]Laboratório Especial de Toxinologia Aplicada – CAT/CEPID, [3] Laboratório de Imunoquímica, Instituto Butantan, SP, Brazil

Introduction: The term atypical enteropathogenic *E. coli* (EPEC) is used to define the EPEC strains that do not have the EAF plasmid. Until now, there are no reported exclusive atypical EPEC virulence factors that could be used to characterize these strains as a new diarrheagenic E. coli pathotype. **Objectives**: In the present study, we analyzed the proteomic profile of outer membrane and periplasmic proteins of typical and atypical EPEC, and compared them looking for the presence of virulence factors that could be related with the pathogenicity of each EPEC group. **Methods**: The typical EPEC strain serotype O55:H6 and the atypical serotype O55:H7 were used in this study. For protein extractions, both strains were grown in Luria broth (LB) and D-MEM. The OMP fractions were obtained using sarcosine extraction, and the periplasmic proteins were obtained using the method of osmotic shock. Western blotting was carried out using hyperimmune sera against the protein extract produced in mouse immunized intraperitoneally with OMP extracts. The protein fractions were analyzed in one-dimension and two-dimensional electrophoresis, and the selected spots were identified using MALDI-TOF-MS methods. The zymogram was applied to detect the presence of proteases in the extracts. **Results**: In the SDS-PAGE analysis of the extractions, we identified a better expression of OMP in typical EPEC strain grown in LB. In the analysis of periplasmic proteins, we identified a better protein expression in atypical EPEC strain grown in D-MEM. The analysis of the OMPs and the periplasmic proteins in one-dimensional electrophoresis did not show significant differences between typical and atypical EPEC that would justify the differences in the pathogenicity of them. Therefore, two-dimensional gels were run. The gels were compared and some of the spots were chosen to be submitted to peptide analysis by the MALDI-TOF-MS method. Both protein extracts showed no proteolytic activity against gelatin. **Discussion**: We are now analyzing the protein spots in 2DE, and identifying the protein of each EPEC fraction, to better elucidate the mechanism of virulence of the EPEC pathotype.

Supported by FAPESP, CNPq/PIBIC

9.19 Growth of *Neisseria lactamica* in four different culture media

Gonçalves BI, Rivera HCB, Schenkman RPF
Centro de Biotecnologia, Instituto Butantan, SP, Brazil

Introduction: *Neisseria lactamica* and *Neisseria meningitidis* are Gram-negative encapsulated diplococci that colonize the upper respiratory tract of humans. They are carried in aerosol of nasopharynx secretions. *Neisseria lactamica* is a commensal microorganism while *Neisseria meningitidis* can be pathogenic causing meningococcal disease. *N. lactamica* is one of the first species from *Neisseria* to colonize the newborn pharynx. It is currently postulated that colonization by *N. lactamica*, that possibly share antigens with *N. meningitidis*, can contribute to the natural development of immunity against *N. meningitidis.* Therefore outer membrane vesicles (OMVs) from *N. lactamica* are potential antigens against *N. meningitidis* and are a potential mucosal adjuvant for a co-administered model antigen. Study of the growth of *N. lactamica* and studies regarding purification and productivity of OMV from *N. lactamica* may provide the key to large-scale culture and OMV production. **Objectives:** The aim of this work was to compare the growth of *Neisseria lactamica* and the yield of outer membrane vesicles (OMVs) in four different liquid culture media. **Methods:** *N. lactamica* was cultivated in a shaker, at 150 rpm, 36 ^{o}C, for eight hours. The culture media tested were: 1) Catlin defined medium without iron and with 1% horse serum; 2) Todd Hewitt broth; 3) BHI (brain heart infusion) and; 4) TSB (tryptic soy broth). Samples were collected every hour. Biomass was determined by optical density at 540 nm of culture broth. OMV were purified from culture broth by centrifugation followed by ultracentrifugation. OMV proteins were measured by the Lowry method. OMV protein profile was determined by SDS-PAGE in gels containing 10% acrylamide. **Results:** *N. lactamica* grew in all culture media. After 8 h culture the OD_{540} reached 2.16 in BHI, followed by 1.23 in Todd broth, 1.21 in Catlin medium and 1.12 in TSB. The OMV yield was 33.25 mg/L in BHI, 27.34 mg/L in Todd, 28.18 mg/L in TSB and 25.89 mg/L in Catlin medium. The electrophoretic pattern was the same in BHI, Todd broth and TSB but in Catlin medium it was different with five more proteins between 53 and 220 kDa. **Discussion:** The best growth of *N. lactamica* and the best OMV yield was obtained in BHI when compared with the other media. However, it has the disadvantage being a medium with compounds of animal origin which is not suitable for vaccine production. The preliminary results suggest that the study of growth in Catlin medium should be continued for a better understanding of the different quantity or quality of the electrophoretic profile since these proteins could be important as potential antigens or adjuvants.

Supported by CNPq/PIBIC, FAPESP

9.20 Suppression of pulmonary allergic inflammation by *Ascaris suum* infection in a mouse model

Mondin R, Araujo CAA, Macedo-Soares MF
Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil

Introduction: Helminth infections have been associated with an important immunomodulatory effect on a host's immune system, impairing the immune response to heterologous antigens, allografts, and infections caused by viruses, bacteria, and other helminths. Furthermore, there is irrefutable evidence that helminth infections can either increase or decrease the allergic reactivity. Parasitic helminths stimulate a type-2 immune response with the production of large amounts of both parasite-specific and non-specific IgE, occurrence of immediate hypersensitivity reactions and recruitment of eosinophils. Despite the increased concentrations of IgE and Th2 cytokines normally associated with asthma, heavily helminth-infected populations exhibit decreased atopy. Several immunoepidemiological patterns indicate a decline in immune responsiveness during infection, which is reversed after removal of parasites by chemotherapy. Negative correlations between immune responses and worm burdens are consistent with both immunosuppression and protective immunity. The type-2 immune response against parasitic helminths, which is functionally protective, establishes at the same time a striking nonspecific immunosuppression that disables the host to set up appropriate responses against other infections or antigens. In the last few years, we have characterized an *A. suum*–derived substance, named PAS-1, which inhibits anaphylactic reactions induced by the helminth's allergenic components and by unrelated antigens by down-modulating cytokines and Tregs. **Objectives:** In the present work, we investigated the effect of *Ascaris suum* infection on airway allergic inflammation in a murine model. **Methods:** BALB/c mice were immunized twice with OVA and alum by intraperitoneal route (days 0 and 7) and challenged twice by intranasal route (days 14 and 21). On day 0, some of these mice were infected with 2500 embryonated eggs of *Ascaris suum*. Two days after the last challenge, airway inflammation was evaluated by serum antibody levels, cellular migration on BAL, eosinophil peroxidase levels (lung tissue and BAL) and cytokine production (BAL). **Results:** Our results demonstrated that non-infected mice, which were immunized and challenged with OVA, had high levels of IgE and IgG1, eosinophilic inflammation and IL-4 and IL-5 production compared with control group (non-infect and non-immunized animals). On the other hand, *Ascaris suum*-infected mice showed a down regulation of antibody production (IgE and IgG1), EPO activity, eosinophil migration (more than 93%) and IL-4 and IL5 cytokines, but enhanced levels of regulatory cytokines (IL-10 and TGF-α). **Discussion:** These data showed that *Ascaris suum* infection down-regulates allergic airway inflammation in a mouse model and this modulatory effect is probably due to IL-10 and TGF-α-dependent mechanisms. A better molecular and functional characterization of helminth molecules that have profound immunomodulatory capacities will offer the prospects of novel therapeutics in limiting or preventing allergic manifestations.

Supporteded by FAPESP, CNPq

9.21 *Ascaris suum* infection suppresses LPS-induced inflammation by IL-10-dependent mechanisms

Titz TO, Araujo, CAA, Macedo-Soares, MF
Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil

Introduction: It is noteworthy that infections with certain helminth parasites stimulate regulatory mechanisms that are correlatively associated with suppression of the host immune responses. In our laboratory, we have demonstrated that *Ascaris suum* worm extract down-regulates antibody production and pulmonary allergic inflammation due the stimulation of regulatory T cell clones. Moreover, we isolated from adult worms a protein (PAS-1) responsible for these suppressive effects. The immune suppression observed with the whole extract or PAS-1 was also obtained with *Ascaris suum* experimental infection. **Objectives:** In this study, we investigated the effect of *Ascaris suum* infection on the leukocyte migration to LPS-stimulated air pouches and the role of IL-10 on this effect. **Methods:** Wild type and IL-$10^{-/-}$ C57BL/6 mice were infected by intragastric route with 2000 *Ascaris suum* embryonated eggs and air pouches were made on their back with sterile air. A week later, cell migration was stimulated with LPS injected inside the air pouches. Control groups received only PBS in their pouches. Three hours after the stimulation, the air pouch exudates were recovered after washing with PBS and centrifuged to obtain the pellet (cells influxed into the pouches) and the supernatant. The pelleted cells were counted and, in the supernantant, pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and IL-10 were determined by ELISA. **Results:** Our results demonstrated that infected wild-type mice demonstrated a significant suppression of the LPS-stimulated leukocyte infiltrate (mononuclear cells and neutrophils) into air pouches and production of TNF-α, IL-1β, IL-6 in comparison to non-infected mice. In contrast, infected wild-type mice produced high levels of IL-10 in relation to non-infected mice. Moreover, the LPS-stimulated leukocyte influx into air pouches and the production of TNF-α, IL-1β, IL-6 in infected IL-$10^{-/-}$ mice was similar to non-infected IL-$10^{-/-}$ mice. **Discussion:** These data demonstrate that *Ascaris suum* infection modulates the LPS inflammation in air pouches by suppressing cell influx and pro-inflammatory cytokines. The evoked mechanism for this suppressive activity is mediated by IL-10 since infected IL-$10^{-/-}$ mice could abolish neither the leukocyte infiltrate into air pouches nor the secretion of pro-inflammatory cytokines.

Supported by CNPq, FAPESP

9.22 *Lafoensia pacari* ethanolic extract selectively regulates the anaphylactic antibody production in mice immunized with the allergenic fraction of *Ascaris suum*

Domingues L[1], Silva SR[1], Favoretto BC[1], Jacysyn JF[2], Takehara HA[1], Albuquerque D[3], Faquim-Mauro EL[1]

[1]Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil; [2]Laboratório de Biologia Celular e Molecular, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP; Brazil; [3]Universidade Federal do Mato Grosso, MS, Brazil

Introduction: A substantial proportion of the Brazilian population makes use of medicinal plants in the form of crude extract, infusion or poultices to provide essential physical healthcare without rigorous scientific knowledge of their efficacy. Thus, medicinal plants have become the focus of study in terms of their pharmacological characterization and biological effects. *Lafoensia pacari* is a species of the *Lythraceae* family and an example of traditional medicinal plant frequently used in the state of Mato Grosso (Brazil) to treat gastric ulcers and inflammation [(1)]. Furthermore, in an experimental murine model of toxocariasis it was demonstrated that *L. pacari* extract exhibited an anti-inflammatory activity induced by the *Toxocara canis* intestinal parasite [(2)]. **Objectives** The aim of this work was to evaluate the effect of the ethanolic extract of *L. pacari* in the anaphylactic antibody production in mice. **Methods:** Groups of male BALB/c mice were immunized with the allergenic antigen from *Ascaris suum* extract (PIII) (50 µg/animal) in Aldrox (7.5 mg/mL) i.p and were treated or not by oral route with *L. pacari* extract diluted in water (1.2 mg/ml) for 28 days. After 21 days of immunization, mice were bled, boosted with PIII i.p. (10 µg/animal) and bled again 7 days later (28 days post-immunization). The antibody production was evaluated by ELISA and passive cutaneous anaphylaxis (PCA). **Results:** The ELISA results showed that mice treated or not with *L. pacari* extract and immunized with PIII produced the same levels of anti-PIII IgG1 and IgG2a. In contrast, mice treated with *L. pacari* extract showed lower levels of IgE and anaphylactic IgG1 antibodies, by PCA. By flow cytometry, no difference was observed in the spleen cell populations of the two groups analyzed. **Discussion:** These results show that the *L. pacari* ethanolic extract exhibits a selective effect on the anaphylactic antibody production, and suggest a potential therapeutic effect of this extract in the control of diseases mediated by anaphylactic antibodies and Th2 responses.

References: 1-Ferreira de Lima MR et al., J. Ethnopharmacol., 11, 2005; 2- Rogerio, A.P. et al, Parasite Immunol., 25, 393, 2003

Supported by CNPq/IBIC

9.23 Presence of anti-rotavirus IgG and IgA in serum samples from healthy Brazilian adults

Ferreira TL[1], Santos SMR[1], Vulcano A[1], Alves PCP[1], Carbonare CB[2], Carbonare SB[1], Tino de Franco M[1]

[1]Laboratório de Imunogenética, Instituto Butantan, SP, Brazil; [2]Laboratório de Imunologia de Mucosas, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil

Introduction: Rotaviruses are well established as being the major etiological agents of diarrhea worldwide. Immunological correlates of protection against natural infection by rotaviruses are not well established. Some studies have correlated serum antibodies against rotavirus in adults with protection and with the probability of infection and illness. Anti-rotavirus IgA and IgG are candidate markers for rotavirus immunity. **Objectives:** The aim of the present study was to establish the assays and to evaluate the titers of serum IgA and IgG anti-rotavirus in healthy adults. **Methods:** MA-104 cell cultures were standardized in roller bottles for SA-11 rotavirus propagation. Afterward, we proceeded with the production of rotavirus antigens by ultracentrifugation purification of SA-11 infected cell supernatant. We analyzed 30 serum samples from healthy Brazilian adults and the detection of anti-rotavirus antibodies was performed by ELISA assay using the purified antigens described above and the anti-rotavirus vaccine produced by Butantan Institute as sensitizing antigens. The titer was determined as the reciprocal of the dilution that provided an absorbance of 0.5. **Results:** We obtained only very low absorbance levels in ELISA assays using the Butantan Institute anti-rotavirus vaccine as sensitizing antigen. Therefore, we used only the purified rotavirus antigens in ELISA assays. The samples showed great variability of anti-rotavirus antibodies. IgG titers varied from 6 to 247.4 and IgA ones from 0.01 to 85.5. **Discussion:** We showed that there is much more anti-rotavirus IgG than anti-rotavirus IgA in our serum samples, and we established the best conditions to detect these antibodies. This work may be important for further studies concerning anti-rotavirus vaccination and the protective roles of immunoglobulin subclasses.

Supported by: FAPESP, PIBIC/CNPq

9.24 Antibodies against major surface proteins from *Neisseria meningitides* B: PorA, PorB, Rmp and Opa/Opc

Rivera HCB, Gonçalves BI, Santos S, Ferreira VRF, Miyaji EN, Oliveira ML, Schenkman RPF
Centro de Biotecnologia, Instituto Butantan, SP, Brazil

Introduction: *Neisseria meningitidis* is the most important cause of meningitis and septicemia worldwide. Against pathogenic serogroups A, C, W135 and Y polysaccharide, vaccines are available whereas polysaccharide from serogroup B is poorly immunogenic since it has a similarity to human cell adhesion molecules. Outer membrane proteins (OMP) from serogroup B are clearly immunogenic in humans and can be obtained from outer membrane vesicles (OMV) released during bacterial cultivation. The major surface proteins in serogroup B are PorA (class 1), PorB (class 3), Rmp (class 4), Opa/Opc (class 5). Immunological and epidemiological studies suggest that the development of natural immunity to meningococcal disease could be related to the colonization of the nasopharynx by commensal *Neisseria* species. **Objectives**: This present research aimed at obtaining and evaluating antibodies against PorA, PorB, Rmp and Opa/Opc from *N.meningitidis* B to be used as a tool in future studies with other *Neisseria* species. **Methods**: *N. meningitidis* B was grown in Catlin medium for 14 h. OMV was purified from culture medium by centrifugation and ultracentrifugation. PorA, PorB, Rmp and Opa/Opc were isolated from OMV in SDS-PAGE (7.5% and 10%). Groups of Swiss mice were immunized subcutaneously with the isolated proteins PorA, PorB, Rmp and Opa/Opc and with OMV. The immunization schedule was three times at three weekly intervals. Antibodies were evaluated by ELISA and Western blotting. ELISA plates were coated with 300 µg OMV per well. For immunoblotting, OMV was separated by SDS-PAGE (10%) and electroblotted to nitrocellulose membrane. Strips were incubated for 2 h in each diluted serum. **Results**: The antibody titers were 1.5 X 10^{-3} (PorA), 1.0X10^{-2} (PorB) and 2X10^{-3} (Rmp). Antibodies against Opa/Opc were not detected. Western blot results show that serum immunized with total OMV recognizes PorA, Rmp, and NadA, Tbp1. Sera obtained from isolated proteins showed reactivity against PorA, PorB and Rmp. **Discussion**: Antibodies against PorA, PorB and Rmp will be useful for future cross reactivity studies among other *Neisseria* species. The serum of animals immunized with OMV showed NadA and Tbp1, two surface proteins that appear in minor amounts in the bacterial surface. Both are more than 70kDa, while all major proteins are less than 50kDa. New immunizations will be performed with these proteins and with Opa/Opc. More studies should be conducted to determine the reactivity and specificity of these antibodies.

Supported by CNPq/PIBIC, FAPESP

9.25 Production and characterization of monoclonal antibodies against rabies virus glycoprotein for diagnosis

Silveira CRF[1], Assumpção GG[1], Brittes AS[2], Faquim-Mauro EL[1], Frazatti-Gallina NM[2], Mourão-Fuches RM[2], Pereira CA[3], Takehara HA[1], Fernandes I[1]
[1]Laboratório de Imunopatologia, [2]Seção de Raiva, [3]Laboratório de Imunologia Viral, Instituto Butantan, SP, Brazil

Introduction: Rabies virus infection causes acute encephalitis that is fatal once symptoms appear, but infection is preventable by post-exposure prophylaxis (PEP). PEP consists of wound care and administration of rabies vaccine alone or combined with rabies immune globulin in some cases. Approximately 10,000,000 people receive post-exposure prophylaxis each year. Detection and quantification of anti rabies antibodies are used for assessment of the effectiveness of rabies vaccines. **Objectives:** The aim of this study was to produce and characterize monoclonal antibodies (MoAb) against rabies virus glycoprotein (GPV). These antibodies will be conjugated to FITC to be used in quantification of anti-rabies antibodies by rapid fluorescent focus inhibition test (RFFIT) and to determine the content of GPV in rabies vaccines. **Methods:** For hybridoma production, lymphoid cells from BALB/c mice immunized with GPV were fused to SP2-0 cell. Screening of hybridomas was carried out by immunofluorescence test (IF) and ELISA. The heavy chain isotype of MoAbs was determined by ELISA, using monoclonal antibodies against different mouse immunoglobulin classes and subclasses. For IF, BHK-21 cells were cultivated in microplates (3.7×10^4 cells/well) in Eagle's medium with 100 FFD_{50}/0.05 mL of rabies virus and incubated at 37°C for 22 h. After fixation of the cells with acetone (80%) the samples containing MoAbs or controls were added and incubated for 1h/37°C. Anti mouse IgG-FITC was added and the microplate incubated for another hour at 37ºC. Afterward, the cells were observed under a microscope to determine the presence of fluorescent foci. The MoAbs were also assayed in S2 (*Drosophila melanogaster* Schneider 2) cells expressing recombinant rabies virus GPV by fluorescent activated cell sorter. The hybridoma supernatants were purified by protein A-affinity chromatography and two MoAbs (clones 1 and 11) were conjugated with FITC. ELISA was performed using GPV or PV/Vero (2 μg/mL) as coating; the plates were blocked and incubated with hybridoma supernatants. Bound antibodies were detected using anti-mouse IgG-peroxidase conjugate and OPD (0.4 mg/mL) plus H_2O_2 as enzyme substrates. **Results:** A total of 19×10^6 lymphocytes were fused with 9×10^6 SP2-O giving rise to 142 clones, from which thirteen hybridomas of IgG1, IgG2a and IgG2b isotypes were positive by ELISA and Immunofluorescence with PV/Vero and challenge standard virus (CSV). These antibodies were designated MAGPV. One MAGPV recognized recombinant rabies virus GPV expressed in S2 cells. MAGPV coupled to FITC was used for direct fluorescence resulting in a positive reaction similar to that obtained with commercial polyclonal antibody. **Discussion:** The monoclonal antibodies produced in this study are suitable for diagnostic purposes since they recognize important epitopes present in PV/Vero and CSV strains as well as heterologous recombinant GPV.

Supported by FAPESP, CNPq/PIBIC

9.26 Immuno-identification of a protein in rat brain cytosol using polyclonal antibody against bradykinin-potentiating peptides (BPPs) precursor protein

Campeiro JD[1], Assakura MT[1], Sant'Anna OA[2], Paes Leme AF[1], Konno K[1], Camargo ACM[1], Hayashi MA[1,3]

[1]Centro de Toxinologia Aplicada - CAT/CEPID, [2]Laboratório de Imunoquímica, Instituto Butantan, SP, Brazil; [3]Departamento de Farmacologia, Universidade Federal de São Paulo, SP, Brazil

Introduction: The bradykinin-potentiating peptides (BPPs) are potent natural inhibitors of the angiotensin-converting enzyme (ACE). This property is very important not only to demonstrate the role of the ACE in blood pressure regulation but also for the development of the first site-directed inhibitor as an anti-hypertensive drug. In 1997, our group was the first to isolate and characterize a cDNA encoding the BPPs precursor protein from snake venom gland. Both the characterization of the BPPs precursor from snake brain also showing a natriuretic peptide at its C-terminus and the demonstration of its expression in snake brain regions correlated to neuroendocrine functions suggested that these peptides could belong to a novel class of endogenous vasoactive peptides. **Objectives:** The aim of the present study was the identification and characterization of endogenous counterpart(s) of BPPs in mammals. **Methods:** With the aim of identifying immunoreactive-related proteins from mammals, the recombinant BPPs precursor protein was obtained by expression in *E. coli* codon plus strain followed by purification using affinity columns. Afterward, the recombinant protein obtained was used to immunize BALB/c mice, and the antiserum raised was used for detection of protein bands from the cytosol of rat brain recognized by the anti-BPP polyclonal antibody. These proteins were isolated and purified by different methods, including 2D-PAGE and HPLC, and their primary structure was determined by mass spectrometry analysis. **Results:** Herein, we describe the expression of the recombinant BPP precursor protein from venom gland of *Bothrops jararaca*, which was used to produce specific polyclonal antibody. The antiserum obtained was shown to be able to specifically recognize the natural and recombinant BPP precursor protein from snake and, surprisingly, it was also capable of recognizing a single protein band in the cytosol of rat brain. We also describe here the isolation and characterization of this immunoreactive protein, which was shown to be the Cu, Zn superoxide dismutase (SOD, EC 1.15.1.1). **Discussion:** The enzyme SOD, recognized by the polyclonal antibody anti-BPP, is an endogenous antioxidant. Interestingly, both SOD and BPP have important roles in blood pressure regulation. In order to understand the relationship between the activity of SOD and BPPs, peptide fragments of SOD, showing BPP-like structural features, were synthesized and bioassays are being performed. Preliminary assays with these peptides suggest that this identified protein might be a potential source for releasing bioactive peptides showing BPP-like pharmacological activity.

Acknowledge: We thank the Proteome Facility of CAT/CEPID for the 2D analysis.
Supported by: CNPq/PIBIC, FAPESP (CAT/CEPID)

9.27 Study of antifungical activity of violacein, a secondary metabolite produced by *Chromobacterium violaceum*

Neto MC[1], Firoozmand LM[1], Cordi L[2], Durán N[2], De Souza AO[1]
[1]Laboratório de Bioquímica e Biofísica, Instituto Butantan, SP, Brazil; [2]Instituto de Química, Universidade Estadual de Campinas, SP, Brazil

Introduction: Fungal infections are a serious health problem around the world. Opportunistic infections caused by *Candida* species have increased greatly and the most commonly used antifungal agents have become ineffective due to the emergence of resistance. Violacein is a secondary metabolite produced by *Chromobacterium violaceum* and displays antimicrobial activity against different pathogens. However, there is no study about its antifungal activity on human pathogenic fungi. **Objectives:** The aim of this study was to evaluate the antifungal activity of violacein *in vitro* and *in vivo* against human pathogenic fungi. **Methods:** The minimum inhibitory concentration (MIC) of violacein was determined with clinical species of *Trichophyton rubrum, C. albicans, Aspergillus fumigatus* and *Cruptococcus neoformans* at concentrations ranging from 16 to 500 μg/mL. In the *in vivo* assay, mice were infected with *C. albicans* by injecting $0.7x10^7$ CFU/animal intravenously. The animals were treated with violacein at 10 mg/kg, and as controls the mice received saline or itraconazole at 10 mg/kg. Drugs were administered orally by gavage at 1, 24 and 48 h after the infections and the animals were observed daily for a week while the survival rates were recorded. **Results:** The MICs of violacein were 250.0, 62.5, 125.0 and 62.5 μg/mL against *T. rubrum, C. albicans, A. fumigatus and C. neoformans*, respectively. In the systemic murine model of candidiasis, 12.5% of the animals that received only saline survived, while in the groups treated with violacein and itraconazole the survival rate was 22.0% and 33.0%, respectively. **Discussion:** The data show that violacein has moderate antifungal activity *in vitro* and *in vivo* against pathogenic fungi. The systemic murine infection induced in this study is very severe but is a good model used to determine if a drug has potential as an antifungal. Although violacein showed moderate activity, new experiments should be performed with higher doses and in a more common infection model.

Supported by CNPq/PIBIC

9.28 Production of anti-*Bothrops jararaca* (*Bj*) fibrinogen antibody in rabbit

Figueiredo AL[1], Vieira CO[1,2], Morais KB[1,2], Tanaka-Azevedo AM[1]
[1]Laboratório de Fisiopatologia, Instituto Butantan, SP, Brazil; [2]Departamento de Fisiologia, Instituto de Biociências, Universidade de São Paulo, SP, Brazil

Introduction: Fibrinogen is a plasma glycoprotein that acts in the final phase of blood coagulation when it is cleaved into fibrin by thrombin. Fibrinogen is composed of three pairs of nonidentical polypeptide chains (Aα, Bβ and γ) interlinked by disulfide bonds. The molecular masses of human fibrinogen chains are 64, 56 and 47 kDa for Aα, Bβ and γ chains, respectively. **Objectives***:* The aim of this study was to produce anti-*Bothrops jararaca* (*Bj*) fibrinogen antibody in order to better characterize *Bj* fibrinogen and to compare it with human fibrinogen. **Methods**: *Bj* fibrinogen was previously obtained through barium chloride *Bj* adsorbed plasma, ammonium sulfate precipitation and gel filtration chromatography (Sephacryl S300 H R 26/60 column). Purified *Bj* fibrinogen was injected into rabbits by the intramuscular route with Marcol-Montanide adjuvant. The animal received three boosters (30, 60 and 90 days after first immunization). The blood was collected from the auricular vein and serum titers were determined by ELISA assay. *Bj* fibrinogen was reduced and alkylated to the S-carbamylmethyl derivatives using iodoacetic acid. Anti-human and anti-*Bj* fibrinogen antibodies were used to visualize Aα, Bβ and γ chains from purified fibrinogen separated by SDS-PAGE and electrotransfered by Western blotting. **Results and Discussion:** The molecular masses of *Bj* chains were 71, 60 and 55 for Aα, Bβ and γ, respectively. Anti-human fibrinogen antibody recognized all chains of *Bj* fibrinogen while anti-*Bj* fibrinogen antibody did not react with human fibrinogen.

Supported by CNPq/PIBIC, FAPESP, CAPES

9.29 A new species of *Calamodontophis* amaral, 1963 (Serpentes, Colubridae, Xenodontinae) from southern Brazil

Cintra LAC[1], Franco FL[1], Lema T[2]
[1]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil; [2]Instituto de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, RS, Brazil

Introduction: The genus *Calamodon* was originally proposed by Amaral (Mem. Inst. Butantan 9: 203-206, 1935) to include a single species, *C. paucidens* Amaral, 1935, based on an individual from the village of São Simão, nowadays Remonta railroad station (29°57'S; 54°57'W), municipality of Cacequi (29°53'S; 54°49'W), state of Rio Grande do Sul, Brazil. However, the generic name was occupied by a fossil mammal, and Amaral (Copeia 3:580, 1963) later proposed the name *Calamodontophis* to replace it. Pagini and Lema described a second specimen from the municipality of Rio Pardo (29°59'S; 52°22'W), Rio Grande do Sul, Brazil. A third specimen from the Araucaria Plateau in the state of Paraná was later identified by Franco *et al.* (Abstr. II Congreso Latinoamericano de Herpetologia, Mérida, Venezuela, p. 82. 1990) as *C. paucidens.* Franco *et al.* (Cuadernos de Herpetoogia, 14: 155-159, 2001) analyzed additional specimens of *C. paucidens* from the Depressão Central do Rio Grande do Sul, Brazil (30°02'S; 52°54'W) and from the Department of Treinta y Tres in Uruguay (32°56'S; 54°26'W). **Objectives**: The aim of this study was to confirm the existence of a new species for the genus *Calamodontophis* and to confirm this genus in the tribe Tachymenini. **Methods**: We examined 14 specimens of *Calamodontophis*, and we counted and measured the scales and cephalic plates. We followed the traditional hemipenial terminology. **Results**: *Calamodontophis ronaldoi* Franco, Cintra & Lema, 2006 was based on two specimens: the holotype (IBSP 55914), from the municipality of General Carneiro (26°25'S; 51°18'W – 983 m a.s.l.), state of Paraná, Brazil, and the paratype (MHNCI 4832), from the village of Três Córregos (25°12'S; 49°38'W – 823 m a.s.l.), municipality of Campo Largo, state of Paraná, Brazil. This species is a representative of *Calamodontophis* that differs from *C. paucidens* mainly by its light longitudinal dorsal line, black blotch stretching from the buccal commissure to the gular region, and black blotch on parietals. The hemipenis of *Calamodontophis paucidens* is very similar to other species of the Tachymenini tribe. Two males of *C. paucidens* (MCP 7834 and MCP 8607) with everted hemipenises were available for this study. Their hemipenis is moderately bilobed, but more bilobed than the other Tachymenini. The bifurcation of the *sulcus spermaticus* is just below the capitulum. Two specimens (MCP 0026 of *C. paucidens* and MHNCI 4832 of *C. ronaldoi*) show a scar of *corpus luteum* typical of viviparous snakes. This is in accordance with our knowledge of viviparity being a characteristic common to all Tachymenini species for which reproductive data are available. **Discussion**: The viviparity condition and the hemipenial morphology place *Calamodontophis* unequivocally within the tribe Tachymenini. Additionally, the probable phylogenetic affinities between this genus and *Tomodon dorsatus* – a Tachymenini – confirms the inclusion of *Calamodontophis* in this tribe.

Supported by CNPq/PIBIC, FAPESP/BIOTA

9.30 Sexual dimorphism, fecundity and female reproductive cycle of *Liophis typhlus* (Serpentes, Colubridae) from southeastern Brazil

Cinaqui-Filho E[1,2], Marques OAV[2], Scartozzoni RR[2]
[1]Universidade Metodista de São Paulo, SP, Brazil; [2]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: The genus *Liophis* (Xenodontinae, Xenodontini) comprises about 40 species distributed from Central America to South America. Reproduction in *Liophis* is extremely variable. Females of some species show seasonal reproductive cycles, but most show continuous reproduction. Information on fecundity and sexual dimorphism are still scarce. **Objectives:** The aim of this study was to characterize the sexual dimorphism of adults, fecundity and female reproductive cycle of *L. typhlus* from southeastern Brazil. **Methods:** We analyzed 177 specimens from Instituto Butantan collection (79 males and 97 females) collected in São Paulo state. For each individual the following data were collected: snout-vent length (SVL), tail length (TL), head length (HL) and body circumference (BC). The diameter of the largest ovarian follicle or oviductal egg, and the number of vitellogenic follicles or eggs were recorded. We considered the males mature if they had a turgid testicle or opaque and convoluted deferent ducts, and the females with oviductal eggs or vitellogenic follicles (>10 mm). The SVL was compared between adult males and females using the *t* test (Student). The other variables were compared between the sex groups by covariance analysis. The TL and BC have the SVL as covariate. The HL has the trunk length as covariate (TRL = SVL-HL). **Results:** Adult females averaged 613 mm in SVL (N = 63; SD = 61.84 mm; range = 537 to 762 mm). Adult males averaged 506 mm in SVL (N = 53; SD = 69.03 mm; range = 358 to 647 mm). Adult females were significantly larger in body size than males ($t = 8.88$; $p << 0.05$). Females were stouter (higher BC) and showed larger HL than males ($F = 5.99$; $p < 0.05$). The TL did not vary significantly between the sexes ($F = 0.08$; $p = 0.77$). The female reproductive cycle was continuous. Vitellogenic follicles (>10 mm) were recorded throughout the year, oviductal eggs were found from January to June and one egg laying was registered in June. Reproductive females (with vitellogenic follicles or eggs) seem to be more frequently collected in the summer and autumn. The fecundity averaged 4.6 eggs/vitellogenic follicle (N = 33; SD = 1.9; range = 1 to 9). The number of eggs/vitellogenic follicle was positively correlated with the female SVL ($r = 0.55$; $p < 0.05$). **Discussion:** Males of *L. typhlus* mature at smaller SVL than females. This trend is common in snakes, including other *Liophis* representatives, and may be related to reproductive attributes (e.g., increased fecundity in females). Females are stouter and have larger heads than males, which could be related to sexual differences in diet. This dimorphism can occur or not in other *Liophis*. The absence of dimorphism in tail length, as observed in *L. typhlus*, is common in *Liophis* and indicates that this characteristic is conservative. The presence of vitellogenic follicles and oviductal eggs throughout the year indicates that the reproductive cycle of *L. typhlus* is continuous. Continuous cycles have been recorded for many *Liophis* species throughout South America being probably a conservative trait in this genus. However, like in other snakes with continuous cycles, *L. typhlus* may display reproductive peaks in favorable periods of the year.

Supported by CNPq/PIBIC

9.31 Inventory of ground dwelling spiders from the Parque Estoril, São Bernardo do Campo, São Paulo, Brazil (Arachnida, Araneae)

Janini CRV, Brescovit AD, Passanha V, Plaza TG
Laboratório de Artrópodes, Instituto Butantan, SP, Brazil

Introduction: Brazil is responsible for 20% of the world's spider fauna. Nevertheless, knowledge on the ground-dwelling species is poor, and studies concerning this particular habitat in the State of São Paulo are rare. **Objectives:** The aim of this study was to inventory the ground- dwelling species of spiders from Parque Estoril, São Bernardo do Campo and to comment on their seasonality patterns. **Methods:** Four samplings, using pitfall traps, were carried out every two months between December/2006 and August/2007. Four previous samples, carried out in the pilot study were also analyzed. In each sampling period, one hundred traps of 500ml plastic cups were installed for 5 days. The content of each cup was considered an independent sample. The resulting material was sorted and identified to the family level. **Results and Discussion:** A total of 942 spiders were collected, 745 adults and 167 juveniles belonging to seventeen families: Sparassidae, Deinopidae, Ochyroceratidae, Ctenidae, Corinnidae, Oonopidae, Tetragnatidae Theridiidae, Linyphiidae, Araneidae, Zoridae, Zodariidae, Lycosidae, Anapidae, Nemesiidae, Salticidae and Pholcidae. Of these, Nemesiidae (2), Ctenidae (5) and Thomisidae (1) were represented only by juvenile species. The average number of spiders per sample was of 2.28. Linyphiidae was the most collected family, with 592 specimens. These results are preliminary, but the abundance of Linyphiidae, a family that occurs in areas heavily modified by humans, indicates that the area studied is an altered environment.

Supported by CNPq/PIBIC

9.32 Characterization of Arthropoda digestive lipase

Filietaz CFT, Lopes AR
Laboratório de Bioquímica e Biofísica, Instituto Butantan, SP, Brazil

Introduction: Lipases (EC 3.1.1.3) catalyze the hydrolysis of triacylglycerols (TAGs), resulting in the release of fatty acids and have been used in numerous applications in food, cosmetics, detergents and pharmaceutical industries. Lipases are ubiquitous enzymes, which are found in animals, plants, fungi and bacteria. Several steps can be distinguished in the processing of dietary lipids, including their emulsification, hydrolysis, micellization, and uptake by enterocytes. Several triglyceride lipases participate in the second step, the hydrolysis of dietary lipids. Although this process is well known in mammals, only little is known of Arthropoda lipase. **Objectives:** We aimed to establish a methodology of lipase assay, measure lipase activity in the midgut of different Arthropoda species, identify the main activities and characterize the most active enzymes. **Methods:** The DMPTB (2,3 dimercapto-1-propanol tributyrate)-DTNB (5,5′-dithiobis (2-nitrobenzoic acid) was standardized and used to measure lipase activity in midgut homogenate samples from: *Nephilengys cruentata, Tityus serrulatus, Gonyleptis saprophilus, Periplaneta americana, Gryllus sp*, *Dysdercus peruvianus*, *Musca domestica*, *Dermestes maculatus*, *Diatraea saccharalis* and *Spodoptera frugiperda.* Molecular mass, pH stability, pH optimum, Km, separation in anion-exchange chromatography are properties which have been studied for these enzymes. **Results**: Lipase activity was measured in midgut homogenate samples from 10 different Arthropoda species. Absolute and specific activities were: *N. cruentata* showed 16 U (one unit of absorbance/min/animal) (0.15 U/μg); *T. serrulatus* 21.2 U (0.66 U/μg); *G. saprophilus* 9.0 U (0.6 U/μg); *P. americana* 15 U (1.7 U/μg); *Gryllus* 2.3 U (0.5 U/μg); *D. peruvianus* 0.4 U (0.65 U/μg); *M. domestica* 1.1 U (2.1 U/μg); *D. maculatus* 0.86 U (0.83 U/μg); *D. saccharalis* 1.7 U (1.1 U/μg) and *S. frugiperda* 5.0 U (1.4 U/μg). *T. serrulatus* lipases displayed pH stability (100% activity at 4°C in a range of pH 3.0-10 and at 30°C a pH 3.5-10); *N. cruentata* (4°C pH 3.0-10.0 and 30°C pH 5.0-9.5); *P. americana* (4°C pH 3.5-10.0 and 30°C pH 5.5-9.5); *D. saccharalis* (4°C pH 5.0-10.0 and 30°C pH 5.5-10), *S. frugiperda* (4°C pH 5.5 –12.0 and 30°C pH 6.5-9.0). Optimum pH of lipase activities was: *T. serrulatus* (a range from 8 to 8.8), *N. cruentata* (8.5), *D. saccharalis* (8.5) and *P. americana* 7.1 and 8.4. Molecular mass determined by gel filtration resulted in a single lipase activity for *N. cruentata* (29.8 kDa) and a major lipase activity for *T. serrulatus* (53.7 kDa). Anion-exchange chromatography indicated only one activity of lipase in samples from midgut homogenates from *T. serrulatus.* Lipase from *N. cruentata* did not interact with anion-exchange chromatography resins. Km values for *T. serrulatus* and *N. cruentata* lipases with DMPTB were: 0.12 mM ± 0.009 mM and 0.12 mM ± 0.019 mM, respectively. **Discussion**: The comparison of all lipase activities measured allowed the identification of the most active enzymes (*T. serrulatus* and *N. cruentata* lipases) and the determination of some lipase properties.

Supported by CNPq/PIBIC, FAPESP

9.33 Characterization of an astacin-like activity from hepatopancreas of the spider *Nephilengys cruentata* (Arachnida)

Bastos AF, Lopes AR
Laboratório Bioquímica e Biofísica, Instituto Butantan, SP, Brazil

Introduction: The digestive peptidases of orb-weaving spiders are necessary not only for consumption of prey, but also for the spider's recycling of its old orb webs into new. The study of spider digestive enzymes spans more than a century but has been sporadic and remains a largely unexplored area. Astacin (EC 3.4.24.21) are metalloendopeptidases from Clan MA family M12, which may be involved in digestion and processing of biologically active peptides. This family of enzymes is found throughout the animal kingdom and also in some bacterial species, and was first found in the digestive system of the Crustacea *Astacus astacu*. This enzyme is known as crayfish small molecule proteinase with a molecular weight of 11 kDa. Some authors indicate the presence of astacin in the digestive juice of spiders. **Objectives:** The aim of this study was to characterize endopeptidases involved in spider digestion homogenate samples from *Nephilengys cruentata* hepatopancreas as well as in elution fractions from anion exchange chromatography and gel filtration, and to identify and isolate astacin in the *Nephilengys cruentata* digestive system. **Methods:** In order to measure and identify different endopeptidases, hemoglobin, casein-FITC, Ac-Ala-Ala-Ala-pNa, Z-FR-MCA were used as substrates. Exopeptidases were also determined. Homogenate samples from *Nephilengys cruentata* hepatopancreas were submitted to gel fitration chromatography or applied to an anion-exchange column. **Results:** The following enzymes were assayed in both samples, HP and DJ, respectively: aminopeptidase (LpNa; 2.4 mU/mg; 3.0 mU/mg), dipeptidase (Gly-Leu, 1.4 mU/mg; 2.0 mU/mg); carboxypeptidase (Z-GlyPhe 3.0mU/mg; 0.3 mU/mg), endopeptidase (carbobenzoxy-PheArg-7-amido-4-methyl-coumarin 11 U/mg; 6.7 U/mg). Cysteine-proteinase had an optimum pH of 3.5 and 4.3. No activity was observed using Ac-Ala-Ala-Ala-pNa as substrate. *Nephilengys cruentata* hepatopancreas contained cysteine-proteinase activity evidenced by activity on hemoglobin and Z-FR-MCA, dependence on cysteine and EDTA in the reaction medium, inhibition by E-64 and lack of inhibition by PMSF. Besides that, an activity demonstrated using casein-FITC as substrate, which showed anomalous migration on a gel filtration column (the major form with a molecular weight of 11 kDa), a pH optimum of 8.0, lack of inhibition by PMSF, E-64 or pepstatin and inhibition by phenanthroline and EDTA, indicated the presence of a metalloendopeptidase also involved in spider digestion. **Discussion:** Some of these properties and literature data indicate that this activity should be classified as an astacin-like enzyme. Separation on an anion exchange chromatography (Hitrap Q column) distinguished two activities (Yield: 800% Purification factor: 7x). Combination of anion-exchange chromatogaphy with gel filtration allowed the isolation of a major protein of 15 kDa visualized by SDS-PAGE.

Supported by FAPESP, CNPq/PIBIC

9.34 Factors with antimicrobial activity in silk glands of *Nephilengys cruentata* (Araneae, Nephilidae)

Bisetto SP[1,2], Silva Jr PI[1]

[1]Laboratório Especial de Toxinologia Aplicada – CAT/CEPID, Instituto Butantan, SP, Brazil; [2]Instituto de Biociências, Universidade de São Paulo, SP, Brazil

Introduction: Spiders produce a family of silk proteins with a wide range of unique physical, mechanical and biological properties; as a result, the use of spider silk has become a study target in biomedicine. Moreover, this silk has been used for many years as material for the treatment of wounds, given its hemostatic capacity. Antimicrobial peptides are potent, broad-spectrum antibiotics, generally between 12 and 50 amino acids long, which are essential factors in the adaptive radiation of arthropods as they work as essential components of their immune system. More than 700 of these peptides have been identified in all living species and they not only work as short contact pathogen killers, but also as immunomodulators, inhibiting lipopolysacharides, inducing pro-inflammatory cytokine production, promoting wound healing, and modulating the responses of dendritic cells and cells of the adaptive immune response. **Objectives:** In order to investigate the presence of antimicrobial peptides among the spider silk proteins as the probable causes for several of its unique properties, we extracted the silk glands from the spider *Nephilengys cruentata*. **Methods:** Different types of glands were extracted in the same manner, but were studied separately. Extracts of the glands were subjected to a pre-purification step by solid phase extraction (Sep-Pak C18, Millipore) and eluted with acetonitrile (80% ACN). The 0% ACN and 80% ACN fractions of each gland were tested for antimicrobial activity by a liquid growth inhibition assay against *Candida albicans*, *Micrococcus luteus* A270 and *Escherichia coli* SBS363. The ACN fractions that demonstrated activity were subjected to reversed-phase chromatography (RP-HPLC), using a Jupiter semi-preparative C18 column. The column effluent was monitored by absorbance at 225 nm and the antimicrobial activity was determined once again by liquid growth inhibition assay. For further purification of the material, the fractions with activity were once again subjected to reversed-phase chromatography (RP-HPLC), this time using a Jupiter analytical C18 column. **Results:** Antimicrobial factors with activity against yeast, Gram-positive and Gram-negative bacteria (*Candida albicans, Micrococcus luteus* and *Escherichia coli*), were found in the 0% ACN fraction of four types of glands and in the 80% ACN eluted acid extraction of three types of glands. The present study focused on the 80% ACN fractions and therefore in ampullate, flagelliform and aggregate glands. Two fractions of the ampullate glands, after RP-HPLC, showed activity against *C. albicans*, while one fraction of flagelliform and one of aggregate glands inhibited growth of both *M. luteus* and *C. albicans*. These fractions will be submitted to new activity assays and the molecular masses will be determined by MALDI-TOF spectrometry analysis.

Supported by FAPESP, CNPq/PIBIC

9.35 Antimicrobial factors from the harvestman *Acutisoma longipes*

Sayegh RSR[1], Daffre S[2], Silva Jr PI[1]
[1]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, Istituto Butantan, SP, Brazil; [2]Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil

Introduction: Arthropods defend themselves against invading microorganisms and parasites through the cellular and humoral immune systems. The cellular reactions are carried out by the blood cells (hemocytes), which immobilize the invaders by phagocytosis and/or encapsulation. The humoral response is performed by constitutive and inducible factors in the hemolymph. These molecules may be involved in recognition, facilitation of cellular immune response or direct antibacterial action. **Objectives**: The objective of this study was to identify antibacterial factors in the hemolymph from the harvestman *Acutisoma longipes* (Gonyleptidae, Opiliones). Harvestmen are an interesting group of arachnids for antimicrobial activity investigation because it is an arachnid that feeds on dead insects, plant juices and on decomposing substances. The characterization of antimicrobial peptides in this class of chelicerates would be of great value to understand the evolutionary aspects of innate immunity in arthropods. Harvestmen *Acutisoma longipes* are very commons in grottos and caves and were collected in grottos around Pedra Grande locality in Atibaia City – São Paulo. **Methods**: The hemolymph (1.6 mL) was collected from prechilled animals by posterior leg articular membrane puncture using an apyrogenic syringe with a sodium citrate buffer solution. The hemocytes were separate from the plasma by centrifugation, the plasma and the hemocytes washed separately in sodium citrate buffer solution were submitted to acid extraction. The acid extract of the plasma and the hemocytes were submitted to a pre-purification in Sep-Pak C18, and was then eluted with different acetonitrile concentrations (5%, 40% and 80% ACN) in 0.046% trifluoroacetic acid (TFA). All the fractions were concentrated in a vacuum centrifuge, reconstituted in 0.046% TFA and separated on a semi-preparative C18 Vydac column using a linear gradient of ACN in 0.046% TFA for the second purification step. The active fractions were then loaded onto an analytical C18 Vydac column for a third purification step. The column effluent was monitored by absorbance at 225 nm and the antibacterial activity was determined by liquid growth inhibition assay. Active fractions from the analytical column were analyzed for purity and molecular mass by mass spectrometry with MALDI/TOF and ESI-MS instrumentation. **Results and Discussion**: Thirteen factors inhibited the growth of the Gram-positive bacteria *Micrococcus luteus* A270: eleven from the plasma and two from the hemocytes. One fraction from the plasma named P3a ($[M+H]^+$ = 2,126 Da) is pure, and its amino acid sequence is under analysis. Another fraction from the plasma named P2 showed the presence of two different molecules: one with $[M+H]^+$ = 2,213 Da, and other with $[M+H]^+$ = 2,342 Da. The low concentration of the molecules obtained from the hemolymph suggests that harvestmen, like insects, need to be challenged to increase the expression of antimicrobial peptides from the immune system. Therefore, the activity found in unchallenged harvestmen seems to be a baseline level.

Supported by FAPESP, CNPq/PIBIC

9.36 Taxonomic and molecular study of species of the genus *Ornithonyssus* (Acari: Macronyssidae) that occur in two biomes of Paraná State

Farias THV[1], Nieri-Bastos FA[1], Labruna MB[2], Arzua M[3], Barros-Battesti DM[1]
[1]Laboratório de Parasitologia, Instituto Butantan, SP, Brazil; [2]Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brazil; [3]Museu de História Natural, PR, Brazil

Introduction: The *Ornithonyssus* genus (Acari: Macronyssidae) is composed of approximately 30 species potentially valid around the world. In the Neotropical region, 11 species are known, including *O. bacoti*. In Brazil, to date, the genus is represented by 8 of them, but molecular analysis showed three new species, which were collected in the last years in the country (CNPq process # 478950/2004-7). **Objectives:** The aim of this study was to determine the species of the genus *Ornithonyssus* collected on wild rodents from the Atlantic Forest, Adrianópolis municipality, and from the Semidecidual Forest, Londrina municipality, State of Paraná, South Brazil, by means of morphology and molecular biology. **Methods:** The specimens were identified through morphological study and also by means of DNA sequences (16S rDNA mitochondrial gene). American samples of *O. bacoti* were also prepared and sequenced molecularly as well as material from rodents collected at the Serra da Cantareira, São Paulo, State of São Paulo. **Results:** Three species of *Ornithonyssus* were found, and they were identified as *O. brasiliensis* (both investigated areas), and *Ornithonyssus* sp1 and *Ornithonyssus* sp2 (found in the Atlantic Forest only). Considering the morphology and DNA sequences, *O. bacoti* does not belong to the Brazilian fauna, and its occurrence in Latin America may be misleading. **Discussion:** Besides the 5 species of *Ornithonyssus* previously reported on rodents in Brazil, the present study contributes two new species, totaling 14 species in the country. Therefore, this represents almost 50% of the worldwide fauna known for the genus.

Supported by CNPq, CNPq/PIBIC

9.37 Ultrastructural modifications on *Crotalus durissus terrificus* venom glands from two non-venom producing snakes

Camargo GG[1], Giannotti, KC[1], Grego KF[2], Sant'Anna SS[2], Carneiro SM[1]
[1]Laboratório de Biologia Celular, [2]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: Venomous snakes are maintained in the Laboratory of Herpetology for the purpose of venom extraction used in snake antivenom production and for research. Venom is collected monthly but sometimes certain snakes fail to produce venom or present a hemorrhagic or purulent secretion, even after appropriate treatment. **Objectives:** The aim of this study was to determine the alterations occurring in the cellular organelles responsible for venom synthesis and secretion in venom glands where venom production is impaired. **Methods:** Venom glands were excised after decapitation of sedated snakes that failed to produce venom for at least 3 months. Part of the gland was fixed and prepared for routine histology; small fragments were fixed, dehydrated and embedded in Epon resin for electron microscopy. Thin sections were stained with uranyl acetate and lead citrate, and examined in a LEO 906E transmission electron microscope. Micrographs of pathological glands were compared with those of normal glands. **Results:** The secretory cells of the control milked C. *durissus terrificus* venom gland was composed of columnar secretory, horizontal cells and mitochondria-rich cells, lying over a basal lamina. Between the secretory tubules there was connective tissue with capillaries and nerve endings. The secretory cells had a prominent rough endoplasmic reticulum (RER) with dilated cisternae with dense intracisternal granules. The Golgi apparatus was well developed and showed immature secretory vesicles. The apical region had microvilli and mature secretory vesicles, many of them in exocytotic process. Mitochondria were observed in the narrow spaces of the cellular matrix. In the secretory cell cytoplasm of the impaired venom glands, the RER cisternae were almost absent or vacuolated. Intracisternal granules were absent or rare; in one of the glands they were numerous and enlarged. Filamentous or granular substances were accumulated in the cellular matrix where swollen mitochondria were occasionally observed. The Golgi apparatus saccules were disorganized and vacuolated. Secretory vesicles were not present in the cells. Some necrotic areas and areas with apoptotic cells were also observed. The loose connective tissue was abundant and could have shown infiltrated heterophils and other cells. No viruses or other pathogenic microorganisms were detected. **Discussion:** The histological and ultrastructural aspects shows striking modifications in the gland parenchyma and in the cells of the secretory epithelium, which have implications in protein synthesis and secretion. Metaplastic modification of the tissue may be due to chronic irritation as produced by manual pressure over the glands during the frequent venom extraction or to some leakage of the venom to the connective tissue. Though microorganisms were not detected, an earlier ascendant infection cannot be excluded.

Supported byCNPq/PIBIC, Fundação Butantan

9.38 Study of the molecular composition of *Phyllomedusa hypochondrialis* skin

Souza KS[1], Assakura MT[1], Jared C[2], Conceição K[1], Antoniazzi MM[2], Camargo ACM[1], Hayashi MA[1,3]

[1]Centro de Toxinologia Aplicada - CAT/CEPID, [2]Laboratório de Biologia Celular, Instituto Butantan, SP, Brazil; [3]Departamento de Farmacologia, Universidade Federal de São Paulo, SP, Brazil

Introduction: The skin of amphibians has important roles in functions that are essential for the survival of amphibians, including breathing, hydration, anti-predator defense, anti-microbial activity, secretion, temperature control, and others. It was demonstrated that the stress of these animals and also handling by humans promote the release of secretions on their skin surface, and that this event is mediated by the nervous sympathetic system. These secretions are normally produced in the amphibian skin in order to interfere in the homeostasis of predators. However, frequently, due to innocent or accidental handling by humans, they can cause poisoning. Such secretions are mainly constituted by bioactive peptides, and the most complex secretions are those produced by neotropical amphibians of the subfamily Phyllomedusinae. The studies of molecular composition of the amphibian's skin have allowed the discovery of a number of new biologically active components, especially antimicrobial and opioid peptides. **Objective**: In the present work, the molecular composition of a monkey tree frog *Phyllomedusa hypochondrialis* skin was accessed by means of a transcriptome analysis of long cDNAs. **Methods**: Unidirectional cDNA library using a freshly collected skin of the Brazilian monkey tigerleg tree frog *Phyllomedusa hypocondrialis* was constructed in lambda ZAP phage. After *in vivo* mass excision step, isolated plasmidial cDNA clones were purified and sequenced using universal primers. Homology search of sequences obtained was performed by using the BLAST ("Basic Local Alignments Search Tool") program. Sequences were then clustered based on homology and predicted physiological function. **Results**: We report here the analysis of more than 500 independent clones containing inserts with average size of 1.3 kb obtained from this directional cDNA library of *Phyllomedusa hypocondrialis* skin. The average readable sequence length was about 500 bp. Of these clones, about 78% were related to sequences previously described, and they were categorized by their biological functions. As expected, most abundant clones were those corresponding to antimicrobial peptides (28%), including the dermaseptins, dermatoxins, demorphins, phylloseptins, and tryptophillin. On the other hand, a surprisingly high number of cDNA sequences studied here were completely novel, showing no similarity with known sequences (about 22%). Beside them, interestingly, the expression of sequences similar to beta-amyloid, serpins, opioid peptides, hormones, enzyme inhibitors, and calcium-binding protein was also demonstrated in this work. **Discussion**: Besides the limited number of analyzed clones for a transcriptome study, in this work, it was possible to isolate cDNA clones coding for all known antimicrobial peptides described to date. The considerable number of sequences showing no homology to any sequence available in the electronic databases suggests that there are still a great number of novel components from this frog skin to be discovered, which was the case, for instance, for the beta-amyloid and calcium-binding protein, described in this frog skin for the first time.

Supported by PIBIC/CNPq, FAPESP (CAT/CEPID)

9.39 Angiotensin IV modulation of melatonin synthesis

Abrahão MV[1], Peres R[2], Amaral F[2], Villela D[1], Moutinho AA[1], Cipolla-Neto J[2], Afeche SC[1]

[1]Laboratório de Farmacologia, Instituto Butantan, SP, Brazil; [2]Departamento de Fisiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil

Introduction: The pineal gland has a circadian rhythm of melatonin (MEL) synthesis and secretion that is imposed by nocturnal noradrenaline release and modulated by many peptidergic systems. In the rat pineal gland, a local renin-angiotensin system (RAS) was described, the astrocytes being responsible for the angiotensin II synthesis and the AT1 receptors, localized in the pinealocytes, for the physiological response. Angiotensin II could be metabolized by aminopeptidases originating angiotensins III and IV. Angiotensin IV (Ang IV) exerts its actions through its proper receptor (insulin-regulated-aminopeptidase - IRAP). There is an important interaction between insulin and Ang IV, because insulin is responsible for the translocation of the IRAP, localized in intracellular vesicles (with the glucose transporter, Glut4), to the plasma membrane. **Objectives:** In order to better understand RAS in the pineal gland, the objective of this work was to investigate the role of angiotensin IV on melatonin synthesis. We studied the effects of angiotensin IV, evaluating the interactions between astrocytes and pinealocytes. **Methods and Results:** Dissociated cells from rat pineal glands were obtained by papain digestion. After the dissociation, the cells (pinealocytes and astrocytes) remained together for 16h, and after that pinealocytes were isolated. When both cellular types were cultivated together for 60h, this preparation was characterized as co-culture. The cells were maintained in DMEM + FBS (10%). Melatonin synthesis concentration-response curves for noradrenaline were obtained for pinealocytes and co-culture, with the maximal responses between 0.1μM and 1μM. AngIV (10nM) increased melatonin synthesis induced by noradrenaline 1μM in isolated pinealocytes, but when in co-culture this effect was not observed. The evaluation of intracellular calcium by confocal microscopy, using fluo4, showed that Ang IV (10nM) induced a calcium increase in pinealocytes. **Discussion:** Ang IV is an important modulator in the rat pineal gland, increasing noradrenergic-induced melatonin synthesis. It is possible that an increase in intracellular calcium is responsible for this effect, which was shown to occur in pinealocytes by AngIV stimulation. Indeed, AA-NAT, the rate-limiting enzyme to the MEL synthesis, is calcium dependent. In co-culture, Ang IV had no effect, which would indicate that, in this situation, AngIV effect was already occurring by the metabolism of AngII, which was possibly induced by noradrenaline.

Supported by FAPESP, CNPq/PIBIC

9.40 Glycogen-induced antinociceptive effect: Possible correlation with leukocyte migration in rat plantar tissue

Nogueira TO[1], Spadacci-Morena DD[1], Pagano RL[1,2], Giorgi R[1]
[1]Laboratório de Fisiopatologia, Instituto Butantan, SP, Brazil; [2]Laboratório de Neurobiologia Celular, Departamento de Fisiologia e Biofísica, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil

Introduction: Many findings demonstrate the involvement of neutrophils in pain response modulation during the inflammatory process. Although, the participation of these cells in nociception is still contradictory, it has been proposed that neutrophils can play a role either in induction or in inhibition of pain response. Our group demonstrated that neutrophils inhibit nociceptive response through calcium-binding protein S100A9 in glycogen-induced peritonitis in mice submitted to the abdominal writing test [(1,2)]. **Objectives:** The aim of the present study was to investigate the neutrophil activities in the control of nociception, in the presence or not, of carrageenin-induced inflammatory hyperalgesia. Further, we investigated the leukocyte migration profile at several times in which pain response was assessed in animals after glycogen intraplantar injection. **Methods:** Male Wistar rats were intraplantarly injected with glycogen solution (Gly 5%, 100µL/animal) or saline, and after different times, the treatment was evaluated using the paw pressure test. In another experimental procedure, rats were injected with Gly concomitant with carrageenin (Cg, 200µg/paw), and after 3 h, peak of Cg-induced hyperalgesia, treatment was assessed in the nociceptive test. Control rats were injected only with Cg. After these procedures, animals were sacrificed in a CO_2 chamber and plantar tissue was collected for the histological analysis. **Results:** In the absence of the Cg-induced hyperalgesia, rats injected with Gly showed antinociception at 2, 3, 4, 6, 8 and 12 h after treatment. This effect was not observed at 1 and 24 h after Gly administration. In addition, Gly injection reversed Cg-induced hyperalgesia and induced an increase in the animal nociceptive threshold (antinociception). Histological analysis demonstrated an increase in the polymorphonuclear cell migration in the initial times after Gly administration. **Discussion:** These results demonstrated that, unlike with carrageenin, which induces hyperalgesia, glycogen induced antinociception when injected into the rat paws. This effect was also observed against carrageenin-induced hyperalgesia. Neutrophils were the predominant cells during antinociceptive effect. Glycogen inhibits pain response in rats evaluated in the paw pressure model in a process dependent probably on the polymorphonuclear cells. This data corroborate our previous results showing the same inhibition in the mouse abdominal writing test. The possible participation of S100A9 protein in these activities is under investigation.

References: 1-Giorgi et al., J. Leukoc. Biol. 64: 214, 1998; 2-Pagano et al., Mediator Inflamm. 4: 36765, 2006

Supported by PIBIC/CNPq, Fundação Butantan

9.41 Glutamate modulation of melatonin synthesis involves interactions between pinealocytes and astrocytes

Moutinho AA[1], Villela DC[1], Peres R[2], Amaral FG[2], Abrahão MV[1], Torrão AS[2], Cipolla-Neto J[2], Afeche SC[1]

[1]Laboratório de Farmacologia, Instituto Butantan, SP, Brazil; [2]Departamento de Fisiologia e Biofísica, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil

Introduction: The mammalian pineal gland is composed mostly of two types of cells: pinealocytes and glial cells. The pinealocytes are secretory cells responsible for melatonin synthesis and secretion. Glutamate is known as a modulator of the melatonin synthesis, acting by reducing serotonin N-acetyltransferase activity and, as a consequence, melatonin synthesis. Glutamate receptors have been found in both pinealocytes and glial cells in the rat pineal gland. **Objectives:** The objective of this work was to investigate the role of glial cells and pinealocytes in the glutamate modulation of melatonin synthesis. **Methods:** Isolated pinealocytes, astrocytes and astrocytes/pinealocytes (co-culture) cultures were used. The cells were maintained in DMEM + FBS (10%). Ionotropic (AMPA – iGluR1 and iGluR3 subunits) and metabotropic (mGluR1, mGluR2, mGluR3, mGluR5, mGluR7) glutamate receptors were characterized in astrocytes and pinealocytes by RT-PCR. Pinealocytes in culture and in co-culture were stimulated by noradrenaline 1μM plus glutamate (100 to 600μM). Melatonin was quantified in the culture medium by HPLC with electrochemical detection. Intracellular calcium was analyzed by confocal microscopy. **Results:** AMPA (iGluR1 and iGluR3), mGluR1 and mGluR3 mRNAs were found in both cell types. Astrocytes also express mGluR5 receptor, and pinealocytes express mGluR2 receptor. Glutamate had no effect on melatonin synthesis in the isolated pinealocytes. On the other hand, when pinealocytes and astrocytes were cultivated together, glutamate reduced melatonin synthesis stimulated by noradrenaline. An increase in intracellular calcium was observed in both cell types induced by glutamate. **Discussion:** The inhibitory effect of glutamate on melatonin synthesis seems to be mediated by the interaction between astrocytes and pinealocytes, which is supported by the presence of glutamate receptors and the calcium response in both cell types.

Supported by: FAPESP, CNPq/PIBIC

9.42 Basic carboxipeptidases-substrates and inhibitors based on the structure of 5-guanido-2-nitrobenzoic acid

Paschoal Jr N, Klitzke C, Melo RL
Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, Instituto Butantan, SP, Brazil

Introduction: Basic carboxypeptidases are metallocarboxypeptidases (CPs) involved in a broad range of the human digestion of dietary proteins, the processing of secreted peptides and proteins, and the regulation of hormone activity in the modulation of protein-protein interactions. The basic carboxypeptidases exhibit a strict specificity for removing C-terminal basic amino acids Arg and Lys from peptides and proteins, and therefore they are referred to as basic metallo-CPs. **Objectives:** The aim of the study was the development and assessment of substrates and inhibitors of basic CPs using the structure of 5-amino-2-nitrobenzoic acid (Gnb) as motif. **Methods:** The synthesis of the new chemical entity Gnb was achieved by guanylation of 5-amino-2-nitrobenzoic acid. This new structure was introduced in peptides by using solid phase peptide synthesis. Characterization of the molecules was achieved by HPLC and mass spectrometry analysis. Spectrofluorimetric and spectrophotometric analyses were used for monitoring and evaluating the enzymatic activities. Hippuryl-Arg was used as substrate for inhibition assays. Carboxypeptidase B (CPB) was used as reference enzyme. **Results:** The synthesis of Gnb was obtained with 90% yield. Gnb was introduced in the synthesis of Abz-Ala-Lys(Gnb)-OH and Abz-Ala-Dab(Gnb)-OH. These Fret-peptides (fluorescence resonance energy transfer peptides) showed a low hydrolysis rate when compared with hippuryl-Arg substrate, but these results can be used in the synthesis of new Fret-peptides for continuous monitoring of CP activities. Because of the low interaction of these substrates with the active site of CPB, we have tested these Fret-peptides, Gnb, Lys(GnB)-OH and Dab(Gnb)-OH as inhibitors and the obtained Ki values were >1 mM. The results were compared to other inhibitors and molecular docking assays in order to confirm the structure-activity relationships. **Discussion:** Rapid and precise monitoring of CP activity is of great importance. However, current methods represent laborious work and are time-consuming. The use of Fret-peptides is a good, accurate and quick way to assess this matter. Moreover, the metabolic importance of CPs as regulators of hormone activity make them a target for the design of new inhibitors.

Supported by FAPESP, CNPq/PIBIC

10. FUNDAP program

10.01 Induction of the anti-phagocytic mechanism by an atypical enteropathogenic *Escherichia coli* (EPEC) sample

Melo KCM, Ruiz RC
Laboratório de Bacteriologia, Instituto Butantan, SP, Brazil

Introduction: EPEC is among the most common bacterial agents in endemic childhood diarrhea in Brazil. The atypical EPEC differs from the typical one by not possessing the EAF plasmid and is considered an emerging pathogen. The increase in diarrhea cases caused by atypical EPEC, as typical EPEC diarrhea cases drop, evokes the capacity of adaptation and pathogenicity that this EPEC subtype is developing. Macrophages can be viewed as either an opportunity or an obstacle for microbial pathogens. Several virulent bacteria have evolved mechanisms to prevent phagocytosis. The interaction of typical EPEC with phagocytic cells inhibits its uptake. This requires a functional type III secretion system and occurs via the inhibition of PI 3 kinase. Developing an anti-phagocytic mechanism seems to improve the colonization of the epithelial cells by enteric bacteria, by delaying the activation of the immune response. **Objectives:** The aim of this study was to investigate the existence of an anti-phagocytic mechanism in atypical EPEC. **Methods:** Macrophage interaction assays were performed with atypical EPEC samples: 7 (O55:H7); 251 (O11:H10); 320(O55:H7) and typical EPEC control (E2348/69) for 10, 30, 60 min infection pulses with different bacterial concentrations. The macrophage cultures were treated with gentamicin and the number of intracellular bacteria was determined. **Results:** Interaction of sample 7 (DO 0.6) with J774 macrophages was smaller both at the 10 min and the 30 min pulses, with 20.3% and 49.9% infection, respectively, while the interaction of 251 sample was 90.9% and 96.3%. Sample 320 infected 76.3% and 81.9% of the macrophages at the same times, and E2348/69, 91.2I% and 94.2%, respectively. At the 10 min infection pulse, 97 % of the macrophages infected with sample 7 showed up to 5 bacteria per cell, adhered or internalized. In the E2348/69 control group, only 20 % of the cells displayed 5 bacteria at most, while 57 % showed more than 15 bacteria per macrophage, in the same infection pulse. At 60 min of infection, 60% of the infected macrophages with sample 7 still showed less than 5 bacteria per cell and only 9 % of the cells had more than 15 adhered or internalized bacteria. On the other hand, 71% of the cells infected with sample E2348/69 displayed more than 15 bacteria per cell. Determination of the number of phagocytized bacteria by macrophages infected with sample 7 was significantly smaller when compared to the control group, infected with typical EPEC at the different infection pulses. The kinetics of interaction with different bacterial concentrations (OD 1.2, 0.6, 0.06 and 0.006) at infection pulses of 10 and 30 min showed that the antiphagocytic effect of sample 7 still occurred at much lower concentrations, such as OD 0.006. In the 10 min infection pulse, both the bacterial interaction and the number of bacteria/cell were less than that observed with the control sample. In the 30 min pulse, only the number of bacteria/cell was lower than with the control. **Discussion:** Sample 7 may have a different anti-phagocytosis mechanism from typical EPEC, which triggers the anti-phagocytosis effect after 2h of interaction, and may not be present at all in samples 320 and 251. This could delay even more the immune response, contributing to intestinal colonization.

Supported by FAPESP, CNPq

10.02 ***Scorpaena plumieri*** **fish venom induces airway inflammation in mice**

Boletini-Santos D[1], Komegae EN[1], Figueiredo SG[2], Haddad VJr[3], Lopes-Ferreira M[1], Lima C[1]

[1]Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, Instituto Butantan, SP, Brazil; [2]Departamento de Ciências Fisiológicas, Universidade Federal do Espírito Santo, ES, Brazil; [3]Departamento de Dermatologia, Universidade Estadual Paulista, Botucatu, SP, Brazil

Introduction: The Brazilian *S. plumieri* fish causes human accidents resulting in severe injuries, characterized by local and systemic effects. **Objectives**: The aim of this work was to study the effect of venom on target organs distant from the inoculation site and on alveolar macrophages *in vitro*. **Methods:** BALB/c mice injected in the right hindpaw with 100 μg of venom were killed after 2, 6, and 24 h for bronchoalveolar lavage fluid (BAL) collection for cell counts and lung tissue collection for cytokine determinations and *zymography*. Alveolar macrophages stimulated with 100 μg of venom for 24h were analyzed for cytokine production. **Results**: Six and 24 h after venom injection, we observed an increased leukocyte influx into BAL, mainly neutrophils and macrophages. The lung inflammation was accompanied by protein extravasation with KC and MCP-1 chemokine production. The distal injection of venom at intraplantar site induced the release of MMP-2 and MMP-9 in BAL and in lung. Finally, the stimulation of alveolar macrophages with venom provoked IL-6 release. **Discussion**: Our results demonstrated that the intraplantar injection of *S. plumieri* venom induced neutrophilic airway inflammation and lung edema. In addition, we showed the importance of alveolar macrophages in the resolution of the lung injury process triggered by *S. plumieri* fish venom.

Supported by FUNDAP, FAPESP

10.03 The role of C-type lectin Nattectin isolated from the *Thalassophryne nattereri* fish venom in local inflammatory response in mice

Pareja-Santos A, Lopes-Ferreira M, Lima C
Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, Instituto Butantan, SP, Brazil

Introduction: Recently, a C-type lectin was identified in the venomous fish *Thalassophryne nattereri,* which was called Nattectin, and shown to have a molecular mass of 15 kDa. **Objective:** The aim of this study was to evaluate the individual role of Nattectin in the induction of local inflammatory reaction. The kinetics of leukocyte recruitment, cytokine and chemokine levels and matrix metalloproteinases 2 and 9 activity after administration of Nattectin in the footpad of Swiss mice was determined. **Methods:** After 2, 6, 24 and 48 h or 7, 14 and 21 days, the animals injected with 10 μg of Nattectin were sacrificed and the footpad was processed for cell suspension collection, and inflammatory mediators were determined in the supernatant of footpad homogenates. The leukocyte cell counts were performed in a hemocytometer using Hema3-stained cytospin preparations. **Results:** At 2, 6, and 24 h or 14 and 21 days after toxin injection, a significant recruitment of leukocytes into footpad of mice was observed, mainly an elevated number of neutrophils. Lymphocytes were recruited at 6 h and 21 days and macrophages only at 24 h. Nattectin induced the synthesis and release of LTB_4 at 6 h, and elevated levels of IL-1β was detected until 48 h, while KC was seen at 7 days. MMP-2 was induced at 2 h with proteolytic activity at 6 h, and 7, 14 and 21 d. MMP-9 was detected 2 and 48 h after injection, but the activity was only observed at 2 h and 21 days. **Discussion:** We demonstrated here that Nattectin, a C-type lectin present in *T. nattereri* venom, is a toxin that induces the release of inflammatory mediators and provokes the recruitment of leukocytes, mainly neutrophils to the site of the lesion. In addition, we can conclude that the lesion induced by *T. nattereri* venom characterized by the absence of leukocytes is probably developed by the major group of toxins, Natterins, which possess kininogenase activity unlike Nattectin.

Supported by FAPESP, FUNDAP

10.04 Expression and characterization of human recombinant anti-crotoxin scFv antibody to be used in immunotherapy

Portes Jr JA[1], Clissa PB[1], Faquim-Mauro EL[1], Magalhães GS[1], Butera D[1], Takehara HA[1], Ramos OHP[2], Fernandes I[1]

[1]Laboratório de Imunopatologia, Instituto Butantan, SP, Brazil; [2]Cientistas Associados, São Carlos, SP, Brazil

Introduction: Crotoxin is the main toxic component of the venom of the South American rattlesnake, *Crotalus durissus terrificus*. It is a presynaptic neurotoxin that contains a phospholipase A2 subunit in a complex with an inhibitory "chaperone" subunit that prevents promiscuous interactions of the phospholipase with phospholipids in membrane surfaces other than its target membrane. Three human anti-crotoxin scFvs (clones 1, 6 and 8) were isolated by combinatorial phage display technology from a naive library of more than 10^{10} scFv clones with *in vivo* neutralizing activity [(1)]. ScFv 6 is the clone that best protected the mice against crotoxin (83%) or whole venom (56%). **Objective:** The aim of this study was to express and standardize the purification protocols and immunochemically characterize scFv 6 produced in bacteria. Furthermore, the scFV 6's sequence would be confirmed by dideoxy-sequencing and compared with previously deposited sequence (Genbank: AJ132608). **Methods:** The production of scFv was induced in *Escherichia coli* (TG1) during 3-4 h at 30°C by the addition of isopropyl β-D-thiogalactoside (IPTG) at a final concentration of 2 mM and directed to the bacterial periplasm. The soluble periplasmic extract was obtained by adding to the cell pellet Tris buffer containing 500 mM of sucrose, maintained in ice for 30 min and centrifuged. ScFv was purified by IMAC using a chelating agarose charged with nickel or cobalt. Different conditions of purification were assayed and the protein content was measured by the Bradford assay. The purity of the final sample was evaluated by SDS-PAGE and silver staining and by Western blotting using mouse anti-His antibody followed by peroxidase-conjugated anti-mouse IgG antibody. **Results:** The yield of scFv using nickel was higher than with cobalt column. Different concentrations of imidazole were employed for binding and elution buffer, and the best results were obtained using 15 and 500 mM, respectively. With immunoblotting, a single band with 30 kDa was observed and the yield, even in the best conditions, was low (175 μg/L). DNA sequence of scFv 6 revealed that it is identical to the previously reported sequence. **Discussion:** ScFv 6 was expressed only in the monomeric form since it resulted in a band consistent with the molecular weight of scFv fused to c-myc and His6 tags, but low levels of expression were achieved. This problem is under consideration, and alternative expression systems have been explored (transformation of other bacterial strains and the use of other recombinant vectors instead of pCANTAB 6). In addition, attempts to improve the affinity and specificity of scFv 6 by site-directed mutagenesis should be carried out using suggestions of the AbEvo (Antibody Evolution) software, under development by BioMinera. AbEvo's algorithm uses 3D information of an antigen-antibody complex to predict advantageous mutations. ScFv 6 with higher affinity and with neutralizing ability may be used for passive immune therapy against *Crotalus* venom poisoning. These human antibodies are expected to cause less adverse reactions than heterologous (horse) antibodies used nowadays.

Reference: 1. Cardoso DF *et al*., Scand. J. Immunol. 51: 337, 2000.

Supported by FAPESP, FUNDAP

10.05 Polysaccharide production from *Haemophilus influenzae* type b in batch and fed-batch cultivation

Silva MR[1], Albani SMF[1], Cabrera-Crespo J[1], Zangirolami TC[2], Takagi M[1], Pradella JGC[1]

[1]Laboratório de Bioprocessos, Centro de Biotecnologia, Instituto Butantan, SP, Brazil; [2]Departamento de Engenharia Química, Universidade Federal de São Carlos, SP, Brazil

Introduction: *Haemophilus influenzae* type b (Hib) is a pathogenic Gram-negative bacterium responsible for causing meningitis worldwide. The capsular polysaccharide composed of polyribosylribitolphosphate (PRP) is the main virulence factor and it is used as an antigen against this microorganism. Vaccines based on polysaccharide must be conjugated to a protein in order to acquire immunological memory, but usually the yield of the final conjugate and purified product is low which result in high cost of this vaccine. The improvement of polysaccharide yield in the preview processes such as cultivation and purification could contribute to improving the cost-benefit ratio and including this vaccine in the World Health Organization Expanded Program on Immunization. **Objectives:** The purpose of this work was to establish a cultivation process that would result in high productivity of polysaccharide. **Methods:** Strain: *Haemophilus influenzae* type b GB3291. Complex culture medium was used, composed of soy peptone (soytone), yeast extract, glucose and growth factor (NAD and hemin). The experiments were carried out in a bioreactor, Bioflo 2000 and Bioflo 3000 (New Brunswick), with 5- and 10-L capacity respectively, 37 °C, pH 7.5 and 0.2 – 1.0 VVM. a) Batch cultivation was carried out in 5 and 10 L of culture medium; when the glucose in the reactor was completely consumed 50% glucose was added to achieve 5g/L. b) Fed-batch was carried out in a 5-L reactor containing 3 L of culture medium in the beginning and then continued as batch experiment. When the glucose was completely consumed, the feed was started with a flow rate of 0.0013L/min. Samples were withdrawn every 2 h and processed. Cell growth was followed by measuring the optical density at 540 nm and dry cell weight. Polysaccharide was measured by the modified Bial method. **Results and Discussion:** The polysaccharide and dry cell weight were: 820 mg/L PRP and 6g/L versus 1.694 mg/L, 15 g/L, respectively for batch and fed-batch. The polysaccharide production was double in fed-batch process compared with the batch one.

Supported by FAPESP, FUNDAP

10.06 Purification of human coagulation factor VIII using different anion exchange resins

Carvalho RR, Jinzenji D, Raw I, Martins EAL, Cheng E
Centro de Biotecnologia, Instituto Butantan, SP, Brazil

Introduction: Coagulation factor VIII (FVIII) is a plasma glycoprotein, whose absence or deficiency causes hemorrhagic disorders characterizing hemophilia A. Hemophilia A is a hereditary disease associated with mutations in the FVIII coding gene, located in the long arm of the X chromosome. Treatment consists of infusions of FVIII concentrates or recombinant FVIII. Most plasma-derived products are produced on an industrial scale by cryoprecipitation, which shows the disadvantages of low recovery and high costs. Alternatively, we propose the use of FVIII purification employing solely chromatographic methods. **Objectives**: The aim of the study was to examine the use anion exchange resins as a first step of purification of FVIII from human plasma. The first resin tested was Q-Sepharose F.F., which displays important features such as a rigid matrix of agarose, minimizing variations in pH and ionic strength. The size of the beads varies from 45 to 165 μm and the flow capacity is 400 – 700 cm/h. **Methods:** Chromatography was performed with direct plasma application in the anion exchange column and using citrate buffer containing different NaCl concentrations for elution. The chromatographic fractions were analyzed using Bradford method for determination of total protein concentration, the chromogenic method for determination of FVIII activity and Western blotting for detection of other proteins. **Results and Discussion:** Results of Q-Sepharose F.F. chromatography indicated that this resin has high FVIII retention capacity, capturing up to 5 column volumes of plasma FVIII. After an intermediate washing, a purification of approximately 70 fold was obtained. Western blot analyses showed that von Willebrand factor, factor IX and factor X are present in all eluted fractions. Interestingly, Factor V, which is structurally very similar to FVIII, was present in all fractions except the Q500 fraction, the FVIII main fraction. In addition, analysis of Q500 in a gel filtration column showed that FVIII activity was present in lower molecular weight fractions, in comparison to the fractions collected in direct plasma purification in the same column. These results indicate that Q-Sepharose F.F. resin demonstrate good selectivity and high capacity of FVIII retention, being, therefore, suitable for the capture step of the purification. However, the presence of FVIII in smaller size protein complexes is not desirable, because in our purification strategy, the next purification step is in a gel filtration column. It is possible that other Q-Sepharose resins of larger beads, such as Q-Sepharose XL and Q-Sepharose Big Beads, would be a better choice for this capture step.

Supported by FUNDAP, CNPq, FAPESP, Fundação Butantan

10.07 Pharmacological characterization of endothelin receptors in the aorta of the non-poisonous snake *Oxyrhopus guibei*

Mesquita LSM, Pellegrini AA, Souza VVF, Borgheresi RAMB
Laboratório de Farmacologia, Instituto Butantan, SP, Brazil

Introduction: The endothelins (ETs = ET-1, ET-2 and ET-3) are isopeptides with strong vasoconstriction action; they occur physiologically in all vertebrate mammals and in some invertebrates, exerting important functions such as regulation of vascular tone and growth of the smooth muscle among a wide variety of functions. The sarafotoxins (SRTXs = SRTX-a, SRTX-b, SRTX-c, SRTX-d) are isoforms of the endothelins and exert their activity by acting on the same receptors of ETs. In mammals, the ET receptors belong to the family of heptatransmembrane protein receptors connected to G proteins and are classified as type ET_A and type ET_B. The ET_A mediate contraction and are localized in the vascular smooth muscle cells. The ET_B receptors can mediate contraction or relaxant effect, depending on their localization. When located in endothelial cells they are called ET_{B1} and they show a relaxant effect; however, when located in the smooth muscle they are called ET_{B2} and mediate a contraction effect. In non-mammals, there are few studies about the ETs receptors. It is known that in the aorta of the poisonous terrestrial serpent, *Bothrops jararaca* (Bj), the contraction effect is mediated by type ET_A and ET_B receptors, and that the ET_A sequence has high homology with that of mammalian ET_A [1]. **Objective:** We aimed to characterize functional ETs receptors, type ET_A and ET_B, in isolated aorta of *Oxyrhopus guibei* (Og). We also aimed to characterize relaxant ET_B receptors, located in endothelial cells of the aorta of the poisonous snake Bj. **Methods:** Cumulative concentration response curve (CCCR) was constructed in isolated intact aorta of the Og for ET-1 (non-selective agonist) and SRTX-c (selective ET_B agonist), in the absence and presence of BQ788 (selective ET_B antagonist). On pre-contracted, intact, isolated aorta of the Bj, the IRL1620 (selective agonist of receptors ET_B), was added at concentrations of 0.01, 0.03 and 0.06μM, to determine the presence of relaxant receptors. The same assay was repeated in presence of selective antagonists of ET_B, BQ788 and IRL1038, and in the presence of L-NAME (NO synthetase inhibitor) and indomethacin (prostaglandin inhibitor). **Results:** The ET-1 contracted Og isolated aorta (pD_2= 8.2±0.1; E_{max}= 0.57±0.28; EC_{50} = 6.57 e-9±2.6 e-9; n=3) and BQ788 was incapable of antagonizing this response. The aorta of the Og snake was not contracted by SRTX-c (CCCR 10^{-10} to 10^{-7} M). These results indicate the nonexistence of ET_{B2} receptors in the Og aorta. IRL1620 relaxed pre-contracted aorta of the Bj suggesting the presence of ET_{B1} receptors. **Discussion:** Our data suggest that in the vasculature of the Og snake, only ET_A receptors contribute to the control of the vascular tonus, while, in the Bj aorta there is mediation by ET_A (previously cloned and partially sequenced) and by both ET_B receptors, contractile (located in smooth muscle = ET_{B2}) and relaxant receptor (located in endothelial cell = ET_{B1}). Thus, we can speculate that the ETs receptors in evolution of the animals are well conserved, but caution should be exercised when comparing data, because possible species differences in the distribution of functional ET receptors can occur.

Reference: 1. Borgheresi *et al*, Biol Med Exp, 231: 729, 2006.
Supported by FUNDAP, Fundação Butantan

10.08 Hippocampal effects of some isolated toxins of *Tityus bahiensis* venom

Ossanai LTT[1], Lourenço GA[1], Nencioni ALA[1], Lebrun I[2], Dorce VAC[1]
[1]Laboratório de Farmacologia, [2]Laboratório de Bioquímica e Biofísica, Instituto Butantan, SP, Brazil

Introduction: In Brazil, one of the most dangerous scorpions is *Tityus bahiensis*. Nevertheless, there are few studies about the effects of *T. bahiensis* venom, mainly on central nervous system. Scorpion venoms are composed of neurotoxins, polypeptides of low molecular weight. In previous studies performed in our laboratory, the venom of *T. bahiensis* was shown to have strong convulsive activity, with distinct characteristics of *T. serrulatus*. Six fractions of this venom were studied and four of them displayed interesting results in the parameters examined (behavior and electroencephalographic changes and neuronal loss). The fraction called P5 was chosen for this work due to its ability to cause behavioral and electrographic convulsion and large neuronal loss. **Objectives:** The objective of this work was to study the effects of the toxins containing this fraction on the hippocampus of rats. **Methods:** The crude venom, with adequate treatment, was applied to a Sephadex G50 column. Chromatographic profile showed six distinct fractions. Toxin fraction P5 was separated on a reversed-phase column by HPLC. The profile revealed five peaks, three of them were used in this study. Four groups of male Wistar rats (n=6) weighing 250g were submitted to stereotaxic surgery to implant cannulas and electrodes in the hippocampus. One day after surgery, the animals were injected with 1µl of solution of toxin 1, 4 or 5 at a concentration of 1µg/µl or control solution in the same volume. After the injections, the animals were connected to a computerized electroencephalographic recorder, and at the same time a behavior analysis was performed for a period of 4h. Seven days after the injections, the animals were sacrificed and perfused. The brains were removed and prepared for histological analysis. **Results:** Toxin 1 induced myoclonus, salivation, discreet increase of secretion, yawning and masticatory movement. Recordings showed electroencephalographic changes while the animal remained immobile. Histological analysis did not display alterations. Alterations observed with injection of toxin 4 were myoclonus, masticatory movement, lacrimal secretion, salivation and often yawning. The short discharges were frequent and concomitant with immobility of the animal, and sometimes, a burst of spikes together with smaller peaks were seen. Histological analysis showed neuronal death in CA1 area ipsi- and contralateral to injection. Toxin 5 provoked yawning, myoclonus and masticatory movements. Recordings remained practically unchanged and discreet neuronal loss was observed in CA1 area ipsi- and contralateral to injection. **Discussion:** The toxins of fraction P5 of *T. bahiensis* scorpion act on the central nervous system modifying behavior and causing neuronal loss. Toxin 4 is more potent than the others; however, based on previous studies, we noted that all the toxins examined contribute to the action of crude venom. Because neuronal loss occurred, we supposed that the toxins act by increasing excitatory amino acid release. Yawning suggests the release of dopamine as a consequence of toxin injection.

Supported by FUNDAP, FAPESP

10.09 Presence of an active vascular angiotensin-converting enzyme (ACE) in two species of Brazilian snakes

Burckhardt PL, Perussi AP, Breno, MC
Laboratório de Farmacologia, Instituto Butantan, SP, Brazil

Introduction: The angiotensin converting enzyme, or kininase II, is a dipeptidyl hydrolase that plays an important role in blood pressure regulation in vertebrate species. It is a rate limited step in the renin-angiotensin system (RAS), by converting the inactive form angiotensin I (AngI) into the active and potent vasoconstrictor peptide angiotensin II. The latter form could be produced via the classical systemic RAS and/or locally via a tissue system. ACE plays also an important biological activity in the kinin-kallikrein system by inactivating bradykinin, which is a potent vasodilator peptide. RAS was identified in mammal species as well as in non-mammals, such as birds, amphibians and reptiles. Our laboratory has characterized the RAS in the terrestrial snake *B. jararaca* (Bj) and the presence of a functional systemic ACE involved with the cardiovascular function in this snake. **Objectives:** The aim of this study was to identify the presence of a locally functional ACE in two arteries of two species of snakes from the Viperidae family, *Bothrops jararaca* and *Crotalus durissus terrificus* (Cdt). **Methods:** Cumulative concentration-effect curves fpr AngI (10^{-10}– 3 x 10^{-6} M) were obtained in aorta and carotid artery rings of Bj and Cdt, in the absence and presence of an ACE inhibitor captopril (10^{-6} M). The –log of the molar concentration that produces 50% of maximum response (pD_2) was calculated. **Results and Discussion:** Captopril shifted to the right the AngI curves in aorta (pD_2 6.72 ± 0.18 to 5.93 ± 0.02, n= 8) and in carotid artery (pD_2 8.34 ± 0.03 to 7.08 ± 0.4, n= 6) of Bj, and also in aorta (pD_2 7.69 ± 0.02 to 6.91 ± 0.03, n= 5) of Cdt. These results show the presence of an active vascular ACE in Bj and Cdt, which could generate a locally bioactive peptide, AngII. The presence of a systemic and tissue ACE in the Bj suggests a similarity with the functional role of this enzyme in mammalian species.

Supported by PIBIC/CNPq, FUNDAP

10.10 Antimicrobial peptides in the plasma of the *Acanthoscurria rondoniensis* spider

Takeuti KCR, Camargo ACM, Silva Jr PI
Laboratório Especial de Toxinologia Aplicada - CAT/CEPID, Instituto Butantan, SP, Brazil

Introduction: Antimicrobial peptides are an extremely diverse group of small proteins that are considered together because of their native antimicrobial activity and are important components of the vertebrate and invertebrate immune system. The widespread occurrence of these antimicrobial substances suggests that they play a role in innate immunity against microorganisms and other pathogens. It has become increasingly clear that due to the development of antibiotic-resistant microbes, antibacterial and antifungal peptides have attracted attention in recent years, in order to find new therapeutic agents. **Objectives:** The objective of this study was to identify antimicrobial peptides in the plasma of the *Acanthoscurria rondoniensis* spider. **Methods:** The hemolymph was collected from the animals by cardiac puncture with an apyrogenic syringe. The hemocytes were removed from the plasma by centrifugation at 800 x g for 10 min at 4ºC. The plasma collected was mixed with acidified water (TFA – 0.05% trifluoroacetic acid) and agitated on ice for 30 min and centrifuged at 16,000 x g. The soluble part was loaded onto classical Sep–Pak C18 cartridges. Three stepwise elutions were successively performed with 5%, 40% and 80% acetonitrile (ACN/ 0.05% TFA) The Sep-Pak fractions were concentrated in a vacuum centrifuge and reconstituted with 0.05% TFA, which was separated by reversed-phase chromatography on a semi-preparative Jupiter C18 column. Elution was performed with different linear gradients of ACN/0.05% TFA over 60 min at a flow rate of 1.5 mL/min. The active fractions were purified by reversed-phrase chromatography on an analytical Jupiter C18 column. The presence of antibacterial activity was determined by a liquid growth inhibition assay against Gram-negative bacteria *Escherichia coli* SBS363, Gram positive *Micrococcus luteus* A270 and *Candida albicans*. **Results:** According to our results, four fractions showed activity against *E. coli* SBS363, while five against *C. albicans*. Among the fractions that had activity, only two were re-purified in an analytical column, and in fractions obtained, one showed activity against *C. albicans*, and two against *M. luteus*. The other fractions will be re-purified and submitted to new bioassays, and the molecular masses of the fractions obtained from reverse-phase chromatography will be determined by MALDI-TOF spectrometry analysis.

Supported by FAPESP, FUNDAP, CNPq

10.11 Bioactive peptide generation by action of *Bothrops jararaca* venom on endogenous substrates

Januario AS[1], Auada AV[1], Ho PL[2], Lebrun I[1]
Laboratório de Bioquímica e Biofísica, Centro de Biotecnologia, Instituto Butantan, SP, Brazil

Introduction: Venoms are a rich source of proteolytic enzymes and serine and metallopeptidases. These enzymes act on several tissues and proteins present in the bitten animal. The present work shows the "in vitro" generation of bioactive peptides after the action of the *Bothrops jararaca* venom on three endogenous substrates: hemoglobin (Hb), immunoglobulin (IgG) and myoglobin (Myo). **Objectives**: The aim of the project was to identify bioactive peptides produced by the action of the venom of *Bothrops jararaca* on endogenous protein substrates. **Methods**: The biological activities of the bioactive peptides produced were carried out "in vitro" in the isolated guinea pig ileum and in anesthetized rat arterial blood pressure, with the crude incubated material and after high performance liquid chromatography (HPLC) isolation. Electrophoresis (SDS-PAGE) of the different steps of the process was also developed, including the determination of the best incubation time. **Results**: The best time was established at 3 h. Active peptides were mainly found in Myo with particular hypotensive effect on the rat arterial blood pressure. **Discussion**: The study suggests that besides the neutralization of the poison that can be achieve with the serum, the generation of these peptides could cause other effects that cannot be neutralized by the serum.

Supported by FUNDAP, Fundação Butantan

10.12 Cloning of genomic sequences that code for part of the metalloproteinase toxins jararhagin and bothropasin from *Bothrops jararaca* venom

Souza AF[1], Souza MMT[2], Ruiz IRG[1]

[1]Laboratório de Genética, Instituto Butantan, SP, Brazil; [2]Faculdade de Medicina, Universidade de São Paulo, SP, Brazil

Introduction: The jararhagin and bothropasin are metalloproteinases present in *B. jararaca*'s venom which have been used as a tool in the inhibition of tumor metastasis. The precursors of these toxins have homologies of 95.2 % in the pro-domain region, 91.2 % in the catalytic domain, 98.9 % in disintegrin-like domain, and 100 % in the cysteine-rich region. **Objectives:** Preliminary experiments were aimed at the characterization and identification of exons and introns starting from genomic DNA and based on published cDNAs of these toxins. **Methods:** PCR *primers* were designed and amplification products were cloned (*pGEM T Easy Vector*) and sequenced (*Big Dye Terminator Vector*). **Results and Discussion:** Two PCR products were detected in agarose gels (bands A and B) suggesting the existence of one gene for each toxin. Sequencing allowed definition of part of one exon, here designated exon 1, consisting of at least 62 bp, followed by intron 1, with about 630 bp. Exon 2 coincides with the beginning of the catalytic domain, and contains 190 bp; intron 2 has about 530 bp (band A) and 340 bp (band B). Exon 3 has 60 bp; intron 3 has 490 bp (band A) and 453 bp (band B). Exon 4 has 70 bp, and intron 4 has at least 140 bp. New *primers* will be designed to confirm these results and to characterize other contiguous regions. These are the first data in the literature on the determination of exon and intron boundaries for these toxins. Clones containing sequences of interest may be used in future experiments to express protein domains particularly involved with the inhibition of tumor cell growth *in vitro* and *in vivo.*

10.13 Snakes from north coast of São Paulo State, Brazil (Reptilia, Serpentes)

Trevine VC, Franco FL
Laboratório de Herpetologia, Instituto Butantan, SP, Brazil

Introduction: The Suborder Serpentes is included within the order Squamata (lizards, amphisbaena and snakes). These animals are found on all continents, except Antarctic. They inhabit the most diverse microhabitats, with periods of activity also being variable. About 2,980 species can be found worldwide. In Brazil alone, there are nine families representing, 78 genera and 350 species, more than 10% of all the species. The Atlantic Tropical Domain includes areas of dense forests (Atlantic Forest), semi-deciduous stationary forests, beach ridge vegetation (restinga) and mangroves. The Brazilian Atlantic Forest is nowadays portrayed by a few and small fragments inserted within plantations, urban areas and pastures. As a result, about 85% of the Brazilian species at risk of extinction are native to this biome. About 140 species of snakes are estimated to occur in Atlantic Forest range, some even endemic, and 80 species are recorded only for the mountains of Serra do Mar. **Objectives**: The aims of the study were: to elaborate a preliminary list of the snakes occurring on the north coast of São Paulo, in the districts of Ilhabela, Caraguatatuba, São Sebastião and Ubatuba; to discuss the richness and relative abundance of species, and for the most abundant ones, to relate seasonal incidence and recruitment; and to perform comparisons between snake communities, regarding distance, geographic area and biome. **Methods**: This survey was carried out by examining the records of the Reception of Snakes of the Herpetological Laboratory at Butantan Institute, records from the "Alphonse Richard Hoge" Herpetological Collection and by bibliographic research. Biogeographic similarity index was used for area comparisons. Results: For the study area, 3398 specimens, 27 genera and 4 families (Boidae (2), Colubridae (44), Viperidae (5) and Elapidae (2) were recorded. The most abundant species were *Bothrops jararaca, B. jararacussu, Micrurus corallinus* and *Liophis miliaris*. *Bothrops jararaca* and *B. jararacussu* were abundant during the whole year, whereas *L. miliaris* and *M. corallinus* showed a reduction in their number of individuals during the colder and drier months. The recruitment pattern indicates a concentration of births in the first semester of the year for *L. miliaris* and *M. corallinus*. Juveniles of *Bothrops* were recorded throughout the year, with a slight tendency of increasing the number of individuals during the rainy season. Eleven typical species of open areas or semi-deciduous forests were added to the available Serra do Mar inventories. Comparative analysis with other snake community surveys showed a great number of species in common with contiguous areas. **Discussion**: The north coast contained the richest species list among all surveys of Atlantic Forest snakes. The prevalence of poisonous species is due to their selection by regular suppliers, sparing the non-poisonous ones. *Bothrops* recruitment could be biased by the slow growth of juveniles during the dry period following birth. The presence of semi-deciduous forests species can be explained by the presence of natural open areas, creating ecological refuges. Contiguous areas tended to show similarity in the recorded number of species; however altitudinal differences, area dimensions and sampling heterogeneity can interfere with these results.

Acknowledgements to Rodrigo Castellari Gonzalez

10.14 Preliminary data on the feeding behavior of *Epicrates cenchria cenchria* in captivity (Serpentes, Boidae)

Citadini JM[1], Iszlaji C[1], Camargo PTM[2], Serapicos EO[3], Puorto G[1]
[1]Museu Biológico, [3]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil; [2]Seção de Répteis, Parque Zoológico Municipal Quinzinho de Barros, SP, Brazil

Introduction: The species *Epicrates cenchria* shows a wide geographical distribution in the Neotropical region, where it can be found from Costa Rica to Argentina. These snakes reach about 1.5m in length; they are constrictors and viviparous, of terrestrial or semi-arboreal habits and nocturnal. The subspecies *E. c. cenchria* can be found in the Brazilian Amazon region. They feed on birds and mammals, and they subjugate their preys by constricting them. **Objectives:** The aim of this study was to describe the feeding behavior of *E. c. cenchria* young during their first year of life. **Methods:** The snakes studied were born at the Parque Zoológico Municipal "Quinzinho de Barros"(PZMQB) in Sorocaba, São Paulo, and they were kept in the Museu Biológico do Instituto Butantan during the period from 9/20/2006 to 7/18/2007. The young were kept in individual plastic containers, covered with corrugated cardboard and with water *ad libitum*. The environmental conditions were monitored, the average temperature was 25.8 °C, and the air relative humidity was 71.23%. The artificial photoperiod was set at 8 hlight and 16 h dark. Newborn and small mice were offered at the beginning of the feeding process, and as the young snakes grew up, the size and weight of the mice were progressively increased, and they were offered food biweekly. Every three months, biometry was performed to follow the development of the *E. c. cenchria* young. The feeding behavior was analyzed, and subdivided into four distinct phases, according to the literature data: localization, approach and strike; constriction; inspection; and digestion. **Results and Discussion:** Preliminary results showed that the perception of the prey and the strike happened right after the prey was put into the maintenance container. During the feeding processes, the majority of the strikes hit the anterior part of the prey's body, followed by medium and posterior parts. Often, one could observe that when the strike hit the anterior part of the body, the snake ingested the prey right after its death, without performing inspection, in other words, without releasing the prey and searching for its head by using the tongue. During the constriction phase, a variation on the number of coils could be noticed; they were normally 1 or 2. The majority of the snakes ingested their preys by the anterior part of the body; the ingestion by the posterior part happened just a few times and hardly ever happened via the middle part. Literature data show that the detection of the prey, especially in boids and viperids, occurs through an association of thermal, chemical and visual stimuli. The capture of the prey by its anterior part was clearly evidenced in our study and it was described for *Elaphe quadrivirgata* and *Bothrops jararaca*, where the authors of these studies reported that this behavior decreases the possibility of a prey's retaliation. The number of coils present during the constriction phase seems to be related to the size of the serpent or to the force applied against the prey's movement. There is a report about a single coil in the feeding behavior of *E. cenchria* and *Boa constrictor*. However, in *E. c. cenchria* we observed a variation on the number of coils, probably due to the prey capture learning process, since only young snakes were studied in this project.

10.15 Morphological analysis and ecological implications of the body integument of *Chaunus ictericus*

Vasconcellos TP[1], Antoniazzi MM[1], Zanotti AP[1], Rodrigues MTU[2], Jared C[1]
[1]Laboratório de Biologia Celular, Instituto Butantan, SP, Brazil; [2]Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, SP, Brazil

Introduction: The species of genus *Chaunus*, until recently named *Bufo* and popularly called toads, are characterized by a relative dry and cornified integument, which allows them to colonize several habitats including forests, savannah and caatinga. Toads are usually large, with predominantly terrestrial and nocturnal activity. Unlike other amphibians, they are able to withstand long periods directly exposed to the air without risk of desiccation. Synapomorphies of the group includes the presence of small warts spread over the body and of a pair of paratoid macroglands behind the eyes, which are used for chemical defence against predators. **Objectives:** We analyzed histologically integument samples along the body of *Chaunus* in order to better understand the relationships between habitat and integumentary adaptations. **Methods:** Samples of 48 integumentary skin sections of *C. ictericus*were were fixed in 10% formalin and prepared for histology in paraffin. The sections were stained with HE and submitted to PAS, Alcian blue, pH 2.5, bromophenol blue and von Kossa histochemical methods. **Results:** On the whole body, including limbs and paratoids, the apical region of the dermis is totally filled by a thick calcified layer. Cutaneous glands are rare and, when present, they are usually mucous. Ventral skin is very similar to dorsal skin, except for the absence of melanocytes and by having a slightly thinner calcified dermal layer. The inguinal skin is formed by typical "verruca hydrophilica," specialized for water intake, and very rich in peripheral vascularization. The dermis of this region also shows a relatively thick calcified layer. The cloacal region differs from the rest of the skin by the common presence of granular glands and of a differentiated glandular type, which is also observed in the skin covering the paratoids. The palmar surface exhibits a thicker epidermis, which is sinuous in the dermis interface. It is very rich in peripheral vascularization and constitutes the only integumentary region without a calcified dermal layer. **Discussion:** Besides the cornified epidermis, the presence of an integumentary calcified armour involving the whole body is possibly an adaptation related to dry environments, constituting a barrier against water loss. On the other hand, the presence of only a few cutaneous glands in the skin can indicate an adaptation to water saving since glands can constitute areas susceptible to water loss by evaporation. Based on this idea, it is reasonable to suppose that, during evolution, there was a tendency for the concentration of skin glands in strategically located regions such as the paratoids and body warts. The observation of a calcified dermal layer in the inguinal region, which is specialized for water intake, is a strong suggestion that, depending on the direction analyzed (external environment/body, or body/external environment), this layer can serve other functions besides acting as a water barrier. The presence of a larger number of glands in the cloacal region can indicate a possible protection against predator attacks when directed to the posterior region of the body.

Supported by CNPq, FUNDAP, Fundação Butantan

10.16 Evaluation of anti-ophidian properties of compounds used in popular medicine

Furlan MS[1], Clissa PB[1], Faquim-Mauro EL[1], Puorto G[2], Fan HW[3], Moura-da-Silva AM[1]

[1]Laboratório de Imunopatologia, [2]Museu Biológico, [3]Laboratório Especial de História da Ciência, Instituto Butantan, SP, Brazil

Introduction: Snakebite is a public health problem in Brazil, and most accidents are caused by *Bothrops* sp. The treatment is based on antivenom therapy, which is efficient against systemic, but not local effects of the venom. Besides that, the victims in some areas of Brazil do not have an easy access to antivenom, so that alternative medicines are often used. Plant extracts have been used for years in the treatment of snakebite poisoning, although only in a few cases has there been a scientific validation of their anti-ophidian properties. The Amazon region is known for having this kind of "popular medicine," and Pau-X (PX) and Específico Pessoa (EP) are examples of natural remedies made from plants and used as alternative treatment against snakebites. **Objectives:** The aim of this study was to evaluate the capacity of PX and EP in neutralizing, *in vitro* and *in vivo*, the hemorrhagic, edematogenic and coagulant activities of *Bothrops atrox* venom, in comparison to *Bothrops* antivenom (BAV) produced by Butantan Institute, using Swiss mice as experimental model. **Methods:** For *in vitro* experiments, PX, EP or BAV were pre-incubated with the venom prior to injection into mice. For *in vivo* experiments, the compounds and BAV were independently injected, so mice were first administered by oral route with PX or EP, or by intravenous route with BAV, before the injection of *B. atrox* venom. For hemorrhagic activity, the hemorrhagic area on the dorsal skin was observed; edema-inducing activity was observed by measuring the thickness of venom-injected paws; and for coagulant activity, the clotting time of the blood from injected animals was observed. **Results:** *In vitro* experiments indicated the capacity of PX, EP and BAV to partially inhibit edema-inducing activity (around 31% inhibition for EP, 41% for PX and 78% for BAV, 2 h after injection). PX was 100% effective against the hemorrhagic activity, as well as BAV. For coagulant activity, only BAV was significantly effective. In *in vivo* experiments, neither PX nor EP was effective against any of the three activities; in contrast to BAV, which was able to inhibit coagulant activity. **Discussion:** Our results suggest that EP and PX show the potential capacity of neutralizing *in vitro* venom local effects, where PX is as effective as BAV in neutralizing venom-induced hemorrhage. However, when administered independent of the venom, only BAV showed efficacy, and only against the coagulant activity. It was suggested that PX could have a metalloprotease inhibitor among its components, as the hemorrhagic activity was neutralized *in vitro*. However, the same result was not observed when the compound was given prior to the venom, showing that the component may not reach the site of venom injection. This work was a preliminary attempt to prove whether or not PX and EP are effective. With our experimental model, we conclude that these compounds are not effective in neutralizing *in vivo* the main biological actions of *B. atrox* venom. However, more studies need to be carried out in order to determine if these and other natural compounds can contribute to the improvement of snakebite treatment.

Supported by Fapesp

10.17 Profile of the visitors of the Museum of Microbiology Laboratory during the July vacation period

Valentini, MTG[1], Camargo, GSR[1], Pereira FF[1], Casadei K[1], Henrique BC[1], Imparato BA[1], Vieira JLA[1], Bizerra AF[2], Inglez GC[1], Tino de Franco M[1]
[1]Museu de Microbiologia, [2]Museu Biológico, Instituto Butantan, SP, Brazil

Introduction: The laboratory from the Museum of Microbiology is a non-formal educational space whose purpose is to stimulate students' scientific curiosity. The activities held in this laboratory involve not only microbiology assays but also the manipulation of scientific equipments and materials. As the laboratory is conceived only for high school students that visit this space during normal school schedule period, we decided to open this space to all the public that visit the Museum during the July vacation period. **Objectives:** Our aim was to evaluate the public profile and their motivation and expectations in visiting the laboratory. **Methods:** Fifty interviews were performed with the public. We used a questionnaire with open and closed questions, collecting data about the person's age, sex, schooling, reason and expectations for the visit, connection between laboratory demonstrated equipment and daily life, etc. The data were grouped and analyzed afterwards. **Results and Discussion:** We showed that most of the public was composed of women with at least a 3rd grade education and children. Some of the visitors could relate some subjects from the laboratory with their daily lives, as microorganisms in non-treated water and the need to wash vegetables and fruits. In addition, some of the visitors reported the intention to look for more information about microbiology and microorganisms after this visitation. Curiosity was the main reason for entering the laboratory and most of the visitors considered the exposition and this initiative excellent. This kind of research can be helpful in enhancing the Museum educational purpose and can also help the Museum educational team to plan other activities.

Supported by FUNDAP, FINEP, FAPESP and Fundação Butantan

10.18 Method for isolating human leukocytes for *in vitro* assays of pathogen-host cell interaction

Szulczewski V, Troiano PER, Cianciarullo AM
Laboratório de Genética, Instituto Butantan, SP, Brazil

Introduction: The diverse methods described in the literature for blood cell separation use commercial substances that, besides the high cost, can also interact with cells in an unpredictable way, compromising the quality of the results. **Objectives:** The aim of this study was to establish a method for isolating human leukocytes to approach the possible alternative pathways of HPV infect cells, through virus like particles interaction (VLPs), with leukocytes from human peripheral blood. **Methods:** Blood samples collected in sodium heparin were centrifuged at 250 x *g*, 5 min, at room temperature. The supernatant containing leukocytes was collected and centrifuged at 265 x *g*, 3 min, at 15°C. The pellet was resuspended in 0.85% NaCl. Total and differential counts were performed in a Neubauer chamber, and smears were stained with Rosenfeld's dye. Cell viability was determined with gentian violet, trypan blue, rhodamine 123, propidium iodide and $DiOC_6(3)$, by light microscopy and by fluorescence with Zeiss EM 510 Meta laser scanning confocal microscope. For ultrastructural assays, the conventional protocol was applied and leukocyte samples were examined with a Zeiss EM 109 transmission electron microscope. **Results:** Cell counting showed 97.5% of lymphocytes; 0.75% of monocytes; 1.51% of polymorphonuclear cells; 0.11% erythrocytes and 0.13% of platelets. Leukocytes stained with Rosenfeld's dye showed preserved morphology, demonstrating a positive correlation with the control blood smears. Rhodamine 123, which interacts exclusively with mitochondria of living cells, was expressed in these cytoplasmic organelles. We observed an intense nuclear labeling with propidium iodide and intact membranes stained with $DiOC_6(3)$, comparatively demonstrating the cellular integrity by the morphology. Similar results were obtained at the ultrastructural level analysis. **Discussion:** We improved the method for obtaining human leukocytes based on the speed and efficiency, which constitute important factors when we have only small volumes of blood available. We developed an easier, faster, low cost and efficient method, preserving cell morphology and viability, adapted for our research necessities.

Supported by FUNDAP, Fundação Butantan, FAPESP

10.19 Sexual dimorphism in six Xenodontinae species

Serapicos EO[1], Janeiro-Cinquini TRF[1], Cintra LAC[1], Merusse JLB[2]
[1]Laboratório de Herpetologia, Instituto Butantan, SP, Brazil; [2]Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brazil

Introduction: Sexual dimorphism is a common characteristic of many species of reptiles; it is related not only to morphology of the reproductive system, but also to different parts of the body. It occurs in a wide variety of phylogenetic lineages of snakes and involves characteristics such as, scale morphology, coloration and ecology (habitat, thermal selection and diet). **Objectives:** The aim of the study was to verify the presence of sexual dimorphism in six species of Xenodontinae. The species studied were *Philodryas olfersii* and *Philodryas patagoniensis*, (Philodriadini Tribe), *Oxyrhopus guibei* and *Phimophis guerrini*, (Pseudoboini Tribe) and *Tomodon dorsatus* and *Thamnodynastes strigatus*, (Tachymenini Tribe). **Methods:** Biometric data was taken from these specimens, and the snakes were then euthanized and deposited in the Herpetological Collection "Alphonse Richard Hoge" of the Herpetology Laboratory of Instituto Butantan. The data obtained were analyzed by statistical methods (Pearson's Correlation and Student *t*-Test) and compared between sexes. **Results:** The results related to the total length and weight indicated the presence of a positive correlation for some species, but there were no meaningful differences between the sexes. The existing correlation between the snout-vent and tail lengths was shown in all species studied. A variation related to the dimorphism in the relative size of the tail was observed between these species. **Discussion:** Various hypotheses try to explain the sexual dimorphism in snakes' tail length. The reason that males have longer tails could be to accommodate their hemipenises and retractor muscles, or the long tail could be a sexual selection, providing mating ability. The relatively smaller tail of the females could be related to reproductive output.

Supported by PIBIC/CNPq