ROUND TABLES

XXX ANNUAL MEETING ON BASIC RESEARCH IN CHAGAS DISEASE - XIX MEETING OF THE BRAZILIAN SOCIETY OF PROTOZOOLOGY - HOTEL GLÓRIA, CAXAMBU, MG, BRASIL - 10-12 NOVEMBER 2003. **Rev. Inst. Med. trop. S. Paulo, 45**(Suppl. 13), November, 2003.

RTCB1 - ORGANELLAR TARGETING AND EXPRESSION ANALYSIS OF *TOXOPLASMA GONDII* HSP90 DURING BRADYZOITE DEVELOPMENT

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Toxoplasma gondii is an obligate intracellular parasite, member of the phylum Apicomplexa that can infect mammals and birds. In humans, *T. gondii* is known to cause transplacental infection that can lead to abortion or severe neonatal malformations and eye disease. Recently, *T. gondii* has emerged as an opportunistic pathogen of major importance in immunocompromised individuals, frequently as a cause of encephalitis. Infection occurs by oral route, and the parasites diferentiate to the asexual tachyzoite form in the small intestine epitelium. In a few weeks, tachyzoites differentiate to the asexual bradyzoite stage, and generate intracellular latent tissue cysts. The asexual cycle occurs in a large number of warm-blooded animals. It involves an equilibrium between the rapidly dividing tachyzoite and the more slowly dividing bradyzoite. The *in vivo* differentiation to the bradyzoite stage is a stress-related response of *T. gondii* to environmental conditions such as the inflammatory response of the host to the tachyzoite stage. The induction of bradyzoite development in vitro has been associated to temperature, pH, and other kind of stressors, all known to stimulate the expression of heat shock proteins (hsps).

Eukaryotic Hsp90 has a highly selective activity in stressed and unstressed cells, where this chaperone is responsible of housekeeping processes controlling cell growth and differentiation. It is also involved in the assistance of misfolded proteins, maturation, intracellular transport and regulated activities of nuclear hormone receptors and other transcription factors, and protein kinases involved in signal transduction and translation control (Review). Hsp90 has been shown to be essential in three model eukaryotic species, *Sacharomyces cerevisiae, Sacharomyces pombe* and *Droshophila melanogaster*, and also to have developmental functions in several organisms.

The hsp90 expression profile of RH UPRT$^-$ *T. gondii* mutant was analyzed under stress (incubation 1 h at 44°C) and bradyzoite formation conditions (tissue culture under CO_2-free incubation). Messenger RNA production was monitored by a semiquantitative RT-PCR assays using *T. gondii* Hsp90 (TgHsp90) and Tg-a-tubulin set of primers. The protein synthesis was analyzed by Western blot with a rabbit anti-TgHsp90 antibody. The results obtained showed an increase in hsp90 mRNA and protein under both, stress conditions and bradyzoite induction. Densitometry analysis showed that the antigen expression increased six and fivefold in stressed tachyzoite and bradyzoite, respectively, compared to the not stressed tachyzoite stage.

The presence of Hsp90 in tachyzoites and bradyzoites was assessed by indirect immunofluorescence assay (IFA) using the polyclonal antibody mentioned earlier. Colocalization analysis was also performed to determine the intracellular localization of the Hsp90 at different parasite stages. The DAPI staining was used to reveal the location of the nucleus and *Dolichos biflorus* lectin, a marker that specifically stains the cyst wall, was employed to check the presence of bradyzoites after CO_2 starvation conditions. In addition to the cytoplasmic localization, which occurred in both parasite stages, HSP90 was detected in the nuclei of bradyzoites. This result was more evident when different slices of the same picture were obtained by confocal analysis, where in the case of bradyzoites the Hsp90 immunolocalization remained in every slice (including the nucleus), while in the case of tachyzoites the nucleus was always an empty space.

To extend our study, the expression level and subcellular localization of Hsp90 was also analyzed in PK cells, a *T. gondii* clone derivative from ME49 strain, which it is not a mutant. In this case these parasites were stimulated to develop into bradyzoite stage using alkaline induction. PK parasites were grown under tachyzoite conditions (normal culture media at 37ºC) or during 3 or 4 days under bradyzoite forming conditions (RPMI/ HEPES, pH 8.1, 5% FBS, 37ºC, CO_2 0.03%). To monitor the bradyzoite development in the cultures used for western blot and IFA analysis, monoclonal antibodies specific to the tachyzoite surface protein SAG1 or to the bradyzoite specific protein P34 were used. By western blot it was observed that the TgHsp90 was overexpressed during bradyzoite development at 3 and 4 days of culture compared to tachyzoites. Intracellular localization of TgHsp90 in PK parasites, by IFA analysis showed similar result to that obtained with RH UPRT$^-$ parasites. The Hsp90 fluorescent signal seems to be present in the cytoplasm of tachyzoites (SAG1 positive/p34 negative), and colocalize with the nuclei (DAPI signal) and cytoplasm of bradyzoites (p34 positive/SAG1 negative).

To further analyze the differences in staining patterns between stages, we studied the localization of TgHsp90 during development from the bradyzoite to the tachyzoite stage. To do this, the bradyzoites released after trypsin digestion were inoculated onto fibroblast monolayer cells. It was observed that the IFA pattern of TgHsp90 remained for 4 hs post-infection in cytoplasm and nuclei of intracellular parasites from encysted bradyzoites inoculated in culture. These parasites still expressed the mature bradyzoite-specific protein p21, but not the tachyzoite specific protein SAG1. After 24 h post-infection some parasites displayed expression of Hsp90 in the cytoplasm and clearly out of the nuclei. These parasites were p21 negative and SAG1 positive (panels U and T) and showed a first round of cell division. It is interesting to note that some single (without cell division), p21 positive, SAG1 negative parasites that had Hsp90 signal colocalizing with DAPI signal inside the nuclei, were coexisting with tachyzoites with the protein out of the nuclei, in the same culture after 24 h post-infection.

Together, these data suggest that the Hsp90 acquires the ability to traslocate into the nuclei when tachyzoites develop into bradyzoites, and to leave the nuclei as soon as the parasites are committed to switch back to rapidly dividing tachyzoites.

The *T. gondii* TgESTzyd42c07.y1 cDNA, that showed high identity with sequences of the hsp90 family, was sequenced. The nucleotide sequence showed that *Tghsp90* is a 2933-bp cDNA encoding a 708 amino acid protein with a theoretical mass of 81.9 kDa. A high degree of amino acidic sequence identity with other apicomplexan parasites like *Eimeria tenella* (81.07%) and *P. falciparum* (74.44%) is observed, although a high degree of similarity is observed with Hsps 90 from plants like *Orsiva sativa* (70.38%) and *Arabidopsis thaliana* (69.81%). Human Hsp90 alpha and beta both display 67.51% of sequence identity with *T. gondii* Hsp90. Analyzing the same data by phylogenetic tree we could see that *T. gondii* Hsp90 is closer to Hsp90 from other apicomplexa and even plants than to human Hsp90. Several structural motifs that are characteristic of Hsp90 proteins are also present in TgHsp90, including the ATP-binding domain which is related to a superfamily of homodimeric ATPases, comprising in addition to Hsp90, DNA gyrase and Histidine kinase proteins. This highly conserved N-terminal domain oh Hsp90 is also the binding site for geldanamycin (GA), a representative of the ansamycin drugs, which specifically target Hsp90. Using the PROSITE data base, the heat shock Hsp90 protein family signature was found in TgHsp90 sequence. Also, a charged aminoacid domain (glutamine/ lysine rich) of unknown function so far, was identified. Other representative sequences of Hsp90 proteins family were found. At the carboxyl terminus, the *T. gondii* HSP90 contains a peptide recognized by tetratricopeptide repeat TRP domains of co-chaperones like hop and immunophilin proteins (see below). Two kind of putative nuclear localization signals (NLS) were found in the *T. gondii* Hsp90 by an analysis of its amino acid sequence with PSORT (http:// psort.nibb.ac.jp/). By sequence similarity with human Hsp90 it could be found, in the TgHsp90, a putative sequence corresponding to the domain that allows the formation of Hsp90 dimer, this structure is located at the C-termini 191 amino acid residues.

In mammals, nearly 100 proteins are known to be regulated by Hsp90. Most of these substrates are involved in signal transduction, and they are brought into complex with hsp90 by a multiprotein hsp90/Hsp70-based chaperone machinery. They consist of a minimal system of five proteins - Hsp90, Hsp70, hop, Hsp40 and P23 - that assembles stable substrate (Pratt and Toft Exp Biol Med 228:111-

133, 2003). In an effort to start to understand some functions and mechanisms of action of Hsp90 in *T. gondii*, we searched in *Toxoplasma* genome project the existence of the extensively studied hsp90/hsp70 based chaperone machinery. It could be identified the most important proteins that generate the heterocomplex: *T. gondii hop* and *p23* genes. Additionally, cDNAs encoding immunophilins, some of which can bind to heterocomplex, likely allowing cytoplasmic-nuclei trafficking of the heterocomplex ans/or activated substrate, were also found in *T. gondii* database.

Dr. Mariana Matrajt (University of Pennsylvania, USA) and Dr. Jean F. Dubremetz (University of Montpellier, France) also participated in this work. This work was supported by an ANPCyT grant (BID802/OC-AR-PICT 05-04831) and ECOS-Sud program.

RTCB2 - ENZYME SECRETION IN *ACANTHAMOEBA* TROPHOZOITES

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Small amoebas of the genus *Acanthamoeba* grow and multiply as phagotrophic trophozoites able to internalize and eventually digest or exocytose inert particles and microorganisms. Trophozoites encyst under unfavorable conditions, thus explaining why *Acanthamoeba* spp. are among the eukaryotic organisms most frequently isolated from the environment. *Acanthamoeba* spp. have been identified as natural hosts of intracellular pathogens (J. Winiecka-Krusnell & E. Linder, Res. Microbiol. 152: 613-619, 2001) and, also, as the causative agents of granulomatous amebic encephalitis (GAE) and amebic keratitis (AK) (F. Marciano-Cabral & G. Cabral, Clin. Microbiol. Revs. 16: 273-307, 2003). GAE is an opportunistic and often lethal infection affecting immunocompromized hosts, while AK affects healthy individuals and may lead to severe corneal damage.

The mechanisms underlying tissue damage and invasion by the amoeba, although still poorly understood, clearly involve local release of cytotoxic substances and proteolytic enzymes. In keratitis, adherence of trophozoites to cornea epithelial cells seems to precede damage of host tissue, suggesting a contact-induced secretion pathway. In addition, proteinases and other lysosomal enzymes are spontaneously released by trophozoites in culture (T. C. Homan & B. Bowers, J. Cell Biol. 98: 246-252, 1984; Alfieri et al., J. Parasitol. 86: 220-227, 2000, and references therein); the constitutively secreted molecules may also facilitate lesion progression and evasion of host's immune response.

We have been interested in identifying and characterizing proteolytic enzymes secreted by *Acanthamoeba* trophozoites. For comparative purposes, secretion of other hydrolytic enzymes (for instance acid phosphatase) is also under investigation. Our study is being conducted with reference (ATCC) and clinical *Acanthamoeba* isolates.

Azocasein assays have shown that trophozoite's lysates predominantly contain cysteine proteinases (CP) optimally active at pH 5.0 and below, and low levels of serine proteinase (SP) activity. In contrast, and compatible with enzyme activation during secretion, in culture supernatants a considerable hydrolytic activity attributed to SPs was detected at pH 6.0 and above. In time course experiments, the release of both proteinase activity and acid phosphatase increased with time while, in parallel, only basal levels (~0.04 % of the intracellular activity released per hour) of lactate dehydrogenase activity could be measured in the supernatants. Secretion of acid phosphatase was shown to increase during phagocytosis of heat-killed yeast.

Proteinase activities secreted by trophozoites were examined in substrate-containing gels and both CPs and SPs were characterized by multiple banding profiles. Differences found between *Acanthamoeba* isolates are reported at this meeting (Ferreira and Alfieri, poster session). In the *Acanthamoeba* isolates examined, the intracellular SPs were activated during electrophoresis, and split the substrate copolymerized in SDS-acrylamide gels. Ongoing studies make use of a biotinylated, irreversible serine proteinase inhibitor to identify intracellular and extracellular *Acanthamoeba* serine proteinases.

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RTCB3 - TRAFFIC OF *TRYPANOSOMA CRUZI* TRYPOMASTIGOTES WITHIN VERO CELLS COLONIZED WITH *COXIELLA BURNETTI*: INVASION, TRANSFERENCE AND … ESCAPE ?

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We have studied the fate of different *Trypanosoma cruzi* trypomastigote forms after invading Vero cells persistently colonized with *Coxiella burnetii. C. burnetii* is the ethiological agent of Q fever and an obligate intracellular bacterium that resides within acidified vacuoles with secondary lysosomal characteristics. When the invasion step was examined by video microscopy, we were surprised by unexpected manners of recently internalized parasites that ranged from reversible circularization, two trypomastigotes that co-localized and migrated around the *C. burnetii* vacuole, and a reluctant entry in the vacuole.

When using EGFP-LAMP1 transfected cells, we could also follow the transference of recently internalized parasites with the labeled *C. burnetti* vacuole. In such experiments, parasites moving within the cell cytoplasm before fusion appeared to release a trail of LAMP-1 material before fusing with the bacterium vacuole.

We also found that persistent *C. burnetii* infection *per se* reduced only tissue-culture trypomastigote (TCT) invasion, whereas raising vacuolar or cytoplasmic pH with Bafilomycin A1 and related drugs, not only affected the distribution of *C. burnetii* but also increased the invasion of both metacyclic and TCT trypomastigotes, when compared to control Vero cells. Kinetic studies of trypomastigote transfer indicated that metacyclic trypomastigotes parasitophorous vacuoles (PV) fuse more rapidly and efficiently to *C. burnetii* vacuoles. The higher TCT hemolysin and transialidase activities appear to facilitate their faster escape from the PV. Endosomal-lysosomal sequential labeling of the PVs formed during the entry of each infective form with EEA1, LAMP-1, and Rab7 revealed that the phagosome maturation processes are also distinct. Measurements of *C. burnetii* vacuolar pH disclosed a marked preference for trypomastigote fusion with more acidic vacuoles. Our results thus suggest that intravacuolar pH modulates the traffic of trypomastigote parasitophorous vacuoles in these doubly infected cells. We also have investigated the behavior of metacyclic trypomastigotes within *C. burnetii* vacuoles beyond 12 hours of co-infection inside Vero cells. We observed that metacyclic trypomastigotes differentiate after 12 hours and amastigotes replicate within *C. burnetii* vacuoles with a pH around 5.5. After 72 hours of infection, there is a predominance of amastigotes forms on the cytoplasm of *C. burnetii* vacuoles. We obtained preliminary evidence that *T. cruzi* amastigotes may escape from the *C. burnetii* vacuole.

Reference:

Andreoli, W.K. & Mortara, R.A. 2003. Acidification modulates the traffic of *Trypanosoma cruzi* trypomastigotes in Vero cells harboring *Coxiella burnetti* vacuoles. *International Journal for Parasitology*, 33: 185-197.

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RTCB4 - THE INFLUENCE OF THE CYTOSKELETON ON *TRYPANOSOMA CRUZI* EPIMASTIGOTE ENDOCYTOSIS

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Trypanosoma cruzi epimastigotes are polarized cells where endocytic organelles have a very stable cytoplasmic localization, spanning from the parasite anterior region, where the cytostome and flagellar pocket, the sites of endocytic cargo entry, are placed, to the posterior end, where reservosomes, the lysosome-like storing organelles, are found. Cargo digestion occurs inside reservosomes, by the action of hydrolases that come from Golgi complex, always localized at the parasite anterior region. As the molecules involved in the endocytic process must travel for such a distance along the parasite body, it is highly feasible that the cytoskeleton would be required not only to maintain organelle position but also to guide transport vesicles.

T. cruzi epimastigotes possess a very special cytoskeleton: a sheath of singlet stable microtubules underneath the plasma membrane, the subpellicular microtubules (SPMT), five to ten microtubules accompanying the cytostome, and filaments supporting a specialized flagellar attachment zone. No other microtubules were ever detected in the parasite cell body. Although actin filaments are known to be essential for endocytosis in yeast and mammalian cells, there is few information concerning their presence in trypanosomatids: actin gene was found and it also seems to be expressed (De Souza et al., 1983. J. Parasitol. 69:138; Mortara, 1989. J. Protozool. 36:8) but no role for actin filaments has yet been described in *T. cruzi*.

Using the uptake of transferrin and peroxidase, labeled with fluoresceine, colloidal gold or radioactive iodine, as a model for endocytosis, we have studied epimastigotes previously incubated with drugs that act on cytoskeleton to evaluate the different participation of microtubules and microfilaments on endocytosis. Oryzalin and cytochalasin D were used as disrupting agents of stable microtubules and actin filaments, respectively. Taxol and jasplakinolide were used as stabilizing agents, for microtubules and microfilaments. Drug concentrations were much lower than those necessary to kill the parasites.

Some ultrastructural alterations were observed after drug treatment, mainly at the cytostome. SPMTs were not altered and the connections between them and endocytic organelles were maintained, even in oryzalin and taxol treated cells. Microtubule disturbing drugs caused a decrease of about 50% in cargo endocytosis. The effects of cytochalasin and jasplakinolide were more drastic, suggesting that, like in other eukaryotic cells, actin plays a fundamental role in *T. cruzi* epimastigote endocytosis. While jasplakinolide-treated cells presented some internalized tracers, cytochalasin treatment completely blocked ligand entry, entrapping cargo at the cytostome and rendering them accessible to exogenous digestion. We demonstrated, for the first time, that epimastigotes do recycle internalized transferrin. Drugs, mainly the stabilizing ones, affected recycling. Degradation of transferrin inside reservosomes was almost completely blocked by cytochalasin.

At present, we can state that epimastigote endocytic organelles are connected to SPMTs, that may guide their positioning. Actin filaments are implicated in the initial events of endocytosis. Transferrin recycling seems to depend on both microfilaments and microtubules.

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RTCT1 - THE TOXIC EFFECTS AND POSSIBLE MECHANISMS OF ACTION OF HEXADECYLPHOSPHOCHOLINE (MILTEFOSINE) AGAINST *TRYPANOSOMA CRUZI*: STUDIES *IN VITRO* AND *IN VIVO*

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Miltefosine (hexadecylphosphocholine), originally developed as anti-tumoral agent[1], is the newest oral effective drug for the treatment of visceral leishmaniasis[2]. Several groups have demonstrated significant antiprotozoal activities of other phospholipid analogues[3], including toxic effects against *Trypanosoma cruzi*[4-6]. In a recent study, we have tested the effects of Miltefosine against strains of *T. cruzi* that are partially resistant (strain Y) and highly resistant (strain Colombiana) to the drug in clinical use Benznidazole. The results indicated that Miltefosine is extremely toxic against the proliferative forms of the parasite (epimastigotes and amastigotes) from both strains[6]. In addition, we have demonstrated that Miltefosine induced, in a dose-dependent manner, the production of tumor necrosis factor alpha and nitric oxide (NO) radicals by infected and non-infected murine peritoneal macrophages in culture. The results suggested that the ether-lipid analogue might activate macrophages *in vitro*. Nevertheless, the cytotoxic effects of Miltefosine against intracellular amastigotes was independent of the amount of NO produced by the infected macrophages since the same dose-response curves for Miltefosine were observed when the NO production was blocked by the NO synthase inhibitor N^G-monomethyl-L-arginine monoacetate[6]. Preliminary *in vivo* studies with both BALB/c and Swiss mice infected with strain Y indicated that oral Miltefosine (25 mg/kg/day) promoted survival and reduced the parasitemia to levels comparable to those observed when Benznidazole (100 mg/kg/day) was used. A comparative histopathological analysis indicated a significant reduction in the area of inflammatory infiltrates in the heart and liver of Miltefosine and Benznidazole treated animals when compared to untreated controls.

In leukemic cells, Miltefosine seems to interfere with the metabolism of phospholipids and with cellular signal transduction pathways, by promoting an inhibition of protein kinase C (PKC) and Na^+, K^+-ATPase[1]. In order to verify if Miltefosine is able to act in a similar way on *T. cruzi*, we have tested its effects on the metabolic incorporation of tritiated palmitic acid into phospholipids[7] and the effects on the ouabain-insensitive and furosemide-sensitive Na^+-ATPase activity of the parasites[8]. Although the results indicate that Miltefosine act as a reversible inhibitor of the *T. cruzi* Na^+-ATPase, there was no clear interference of the drug in the metabolism of phospholipids when analyzing the radio labeling phospholipid profiles by 1D and 2D TLC. To get additional evidences about the possible mechanisms of action of Miltefosine against *T. cruzi*, we have pre-incubated culture-derived trypomastigotes or murine peritoneal macrophages with the ether-lipid analogue prior to infection. The results suggest that Miltefosine may accumulate inside the parasites or macrophages retaining its cytotoxic properties against the parasites and/or inhibiting protein kinases known to be involved in the phagocytic route of parasite entry.

Supported by CNPq, FAPERJ, IFS and TWAS

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RTCT2 - BIOLOGICAL AND BIOCHEMICAL ASPECTS OF THE ANTI-*LEISHMANIA* CHEMOTHERAPY

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To find a new drug against parasites is important to consider the biological and biochemical aspects, knowing the peculiarity of each species. In the case of *Leishmania* it is necessary to know the biology of the parasite, the infective capacity (amount of metacyclic forms), growth conditions (pH, temperature, amount of sera). Also, it is important to evaluate the toxicity of the compound and the effect on the interaction parasite x host cell. In parallel, the study of metabolic pathways, occurring in a different way in parasite and host cell, will allow the definition of a drug target. Among those pathways, our group have been studying in *Leishmania (L.) amazonensis* (MHOM/BR/77/LTB0016 strain) the NO production, the isolation and characterization of a constitutive nitric oxide synthase cNOS, a redox system involving trypanothione / trypanotione reductase and the phosphorylative activity of some kinases, in particular the cAMP-dependent kinase (PKA), which regulate the NOS activity. In parallel, we have been studying several N, N´-diphenyl benzamidine derivatives against promastigotes and axenic amastigotes of *L. (L.) amazonensis,* and we found that the most effective was the one with a methoxylated substituent. Using this compound it was evaluated the in vitro toxicity and the effect on the interaction parasite x mice peritoneal macrophage. It was also realized a treatment trial using a BALB/c model. It was observed that the methoxylated amidine was able to inhibit the parasite growth (ICs_{50}/24h = 14 ± 1.4 mmol/L and 280 ± 24 mmol/L for promastigotes and axenic amastigotes, respectively) (Canto-Cavalheiro et al., 1997; 2000). Also, this compound induced a decrease in the amount of amastigotes within the macrophage and did not show any toxic effect against the host cell (Temporal et al., 2002). The treatment through intralesional and topic ways showed that the methoxylated amidine was able to avoid the infection in mice treated and followed up for four weeks after injection. Studies concerning the potential targets for this derivative showed that these compound decreased the NO production and TR activity in both promastigotes and axenic amastigotes of *L. (L.) amazonensis* (Castro-Pinto et al., 2003; Genestra et al., 2003 a, b, c). Corroborating the data of the in vitro toxicity, it was observed that the activity of glutathione reductase (GR), the redox system equivalent to TR in the host, was not affected by the drug (Castro-Pinto et al., 2003). Furthermore, the phosphorylative activity of PKA, was significantly diminished in the presence of the compound, confirming the decreasing in the NOS activity (Genestra et al., 2001). In all experiments, Pentamidine Isethionate was used as reference drug and even being more effective against promastigotes and axenic amastigotes of *L. (L.) amazonensis,* it was observed a high toxicity against the host cell, besides the inhibitory capacity against the enzymes assayed and the NO production being smaller, comparing to the methoxylated amidine. Based in these data, we must conclude that *L. (L.) amazonensis* is perfectly prepared to evade the toxic metabolic, both nitrogenated and oxygenated, produced by macrophage to avoid the infection and demonstrate the importance of a compound which have as targets those metabolic pathways (NO/NOS, PKA and T/TR) that are necessary for parasite survival.

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RTCT3 - THE INTERPRETATION OF *T. CRUZI* ANTIGENS PRESENT IN MYOCARDIAL INFLAMMATION IN CASES OF CHRONIC CHAGAS' DISEASE.

Maria de Lourdes Higuchi

Knowledge of the exact role of the parasite in the pathogenesis of chronic chagasic cardiopathy appears extremely important to guide potential therapeutic strategies.

The relative lack of parasites in the myocardium during the chronic phase originated many autoimmune theories, of both humoral and cellular origin[1,2,3]. Common antigens between *T. cruzi* and human myocardial fibers, auto-reactive T cells specific to heart or nerve tissue antigens have been demonstrated in experimental animals and patients[4,5,6]. These autoimmune theories suggest that the myocarditis perpetuates independently of the presence of the parasite[7]. However, only autoimmunity would not explain the multifocal nature of the myocarditis and the preferential location of fibrosis in certain regions such as the apical or the posterior left ventricular wall in chronic chagasic cardiopathy. Moreover, frequent positive xenodiagnosis during the chronic phase of Chagas' disease and during episodes of reactivation in immunocompromised patients (by AIDS, neoplasia or cardiac transplant) has provided evidence that the parasite is present in the chronic phase under active control of the immunological system of the host[8].

Endomyocardial biopsies from patients in different clinical stages of Chagas' disease have provided new insights on some important questions, in particular on the inflammatory process and the development of heart failure. According to these observations, chronic chagasic cardiopathy may be considered a progressive, fibrotic disease in which myocardial inflammation plays a fundamental role[9,10,11]. Studies on myocardial biopsy fragments from patients with chronic chagasic myocarditis have demonstrated that the inflammatory infiltrate was mainly composed of T cells, with a predominance of $CD8^+$ T cells[12,13]. $CD4^+$ T cells were present in lower numbers and were only mildly stained compared to the CD8+ T cells, suggenting a certain degree of immunosuppression in this phase of the disease. Comparison of endomyocardial biopsies in acute and chronic phases demonstrated that patients in acute phase present 100% of myocarditis and 58% of *T.cruzi* antigens, whereas in patients in the chronic phase these values were reduced to 45% of myocarditis and 0% of *T. cruzi* antigens[14]. The lack of parasite antigens in biopsy material from patients in the chronic phase is expected as, according with which we will describe latter, it seems necessary to examine several different sections of the heart to detect the parasite in this phase of the disease.

The presence of *Trypanosoma cruzi* in the chronic phase of the disease has been already observed in early descriptions[15] and has been emphasized later by other authors[16,17]. Nevertheless, the number of parasites is disproportionately low in relation to the intensity of the myocarditis and whole myocardial fibers containing parasites do not elicit inflammation. *T. cruzi* Ags were detected in 100% of hearts from chronic chagasic patients that died due to heart failure when several samples of the myocardium were analyzed, but without direct correlation between the amount of *T.cruzi* Ags and the intensity of the inflammatory infiltrate. A significant association was found with the presence of *T. cruzi* Ags and inflammation. Parasite Ags probably work as a trigger for the hypersensitive response against the myocardial fibers. Jones et al.[18] described a high incidence of *T. cruzi* DNA using the PCR technique in myocardial fragments exhibiting significant inflammation.

An up-regulation of ICAM-1 and induction of VCAM-1 expression on capillaries and venules was present in patients with chronic chagasic cardiomyopathy[19].

Class I and class II MHC antigens were also up-regulated in the myocardium of patients with chronic chagasic cardiomyopathy, favoring inflammation. Such over-expression was not present in the myocardium of patients with idiophatic dilated cardiomyopathy that did not present myocardial inflammation. Since inflammatory cytokines have been demonstrated in inflammatory foci in hearts of patients with chagasic cardiomyopathy, they are probably responsible for the up-regulation of adhesion molecules and MHC antigens. Indeed, over-expression of

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adhesion molecules on the endothelium is probably very important to perpetuate inflammation and the up-regulation of class I MHC antigens on myocytes could represent a target for $CD8^{+}$ T lymphocyte adhesion, promoting direct cytotoxicity.

Myocardial lymphocytes and macrophages may favor the development of fibrosis by production of cytokines and growth factors. Regarding some growth factors, we have observed a strong correlation between numbers of PDGF-A+ and PDGF-B+ cells and lack of correlation between PDGF-A and TGF-β1; PDGF-A and GM-CSF; and between PDGF-B and TGF-β1. GM-CSF and TGF-β1, which are considered important elements for the immune response against *T. cruzi* parasites, were present in very scarce amounts[20].

A severe microvascular dilatation was observed in chagasic hearts, but not in idiopathic dilated cardiomyopathy[21]. The lack of arteriolar contraction might be due to the presence of vasodilator substances induced by the inflammation and/or by the parasite. This may cause a impaired myocardial irrigation in distal areas of the coronary branches. Such a low blood pressure perfusion should be present mainly at the watershed zones between two main coronary artery branches. The resulting ischemia would explain fibrotic lesions such as the apex aneuriysm and fibrosis of the posterior lateral wall of the left ventricle. This could be also an explanation for the fibrotic segmental lesions in the conduction system which is doubled irrigated.

In summary, chronic cardiac Chagas' disease seems to represent the result of a close interaction between the host and the parasite, causing different clinical pictures: patients with a good immune response may adequately circumvent the parasitic infection and the individual will develop the indeterminate form. Deficient immune response of the host or a high initial parasitemia may favor immune imbalance that will favor development of permanent inadequate response of immunological response against the parasite. The inflammatory response, which is probably recurrent, undergoing periods of more accentuated exacerbation, is most likely responsible for progressive neuronal damage, microcirculatory alterations, heart matrix deformations and consequent organ failure.

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RTCT4 -PHOSPHOLIPIDS OF THE MALARIA-INFECTED ERYTHROCYTES: PECULIARITIES AND PHARMACOLOGICAL IMPLICATIONS

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Membrane biogenesis participates in the production and homeostasis of the various subcellular compartments needed by *Plasmodium* throughout its development. Phospholipids (PLs) are the major lipids of the intraerythrocytic parasite since absence of significant amounts of cholesterol or of other lipids has been reported. These newly synthesized PL molecules are very abundant (up to5

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times the host cell content) and very classical with high amount of phosphatidylcholine (PC) and phosphatidylethanolmine (PE) (> 80 % of total) and phosphatidylinositol. On the other hand, metabolic pathways which provide these PL are of a bewildering variety. Indeed, for the production of PC and PE, all the known metabolic pathways present in bacteria, or eukaryotes, even some restricted to plants, are gathered in the parasite. These pathways have been characterized and quantified, and a number of genes cloned and their products characterized. The *Plasmodium* Data Base now provides most of the other expected enzymes. We have indication that the major metabolic pathways are not interregulated contrary to yeast, indicating that they probably exist in specific metabolic compartments whose structures remain to be identified. Finally, it also appears that the erythrocyte membrane is largely remodeled with respect to its molecular composition but maintains a constant level of its major lipid classes (cholesterol or PL classes), probably for its stability. Tools are now needed to dissect the lipid composition of the different organelles and membranes of the parasite all its developmental stages. Also of interest is the role in signaling. Actors mediating their own intracellular dynamics deserved not being ignored.

Besides, PLs represent great potential for future antimalarial pharmacology. One approach targeting the *de novo* PC biosynthetic machinery is in advanced stages with compounds that successfully cure malaria in monkeys at low doses and that indicate excellent apparent therapeutic indices (related to lethal doses). Other chemotherapeutic approaches might be validated in the future due to interesting specific features of the plasmodial enzymes.

RTE1 - MOLECULAR PHYLOGEOGRAPHY AND TRIATOMINE SYSTEMATICS: A PUBLIC HEALTH PERSPECTIVE.

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The phylogeographic structure of the closely related triatomine species *Rhodnius prolixus* and *R. robustus* is presented based on a 663 bp fragment of the mitochondrial cytochrome *b* gene. Twenty haplotypes recovered from 84 samples examined, representing 26 populations from 7 Latin-American countries, were aligned and subjected to phylogenetic analysis. The resulting phylogenetic tree is composed of five major reciprocally monophyletic clades, one representing *R. prolixus* and four representing *R. robustus*. While *R. prolixus* is a very homogeneous assemblage, *R. robustus* has deeper clades and is paraphyletic, with the clade comprising *R. robustus* from Venezuela (Orinoco region) more closely related to the *R. prolixus* clade than to the other *R. robustus* populations from the Amazon region. The *R. robustus* paraphyly was further supported by the analysis of a nuclear gene (D2 region of the 28S RNA) for a subset of specimens. The data support the view that *R. robustus* represents a species complex. The application of a standard calibration for insect mitochondrial molecular clock indicates that levels of sequence divergence between clades within each region are compatible with a Pleistocene origin. Nucleotide diversity (ð) for all *R. prolixus* populations was extremely low (0.0008) suggesting that this species went through a recent bottleneck, and was subsequently dispersed by man.

Implications for vector-control

Rhodnius prolixus is the primary Chagas disease vector in Venezuela, Colombia and parts of Central America. The main reason why it is such a good vector is because it is essentially domestic throughout most of its range. Thus, once a village is treated with insecticide and becomes triatomine-free, there will be no great risk of reinvasion of treated premises from sylvatic foci. Hence, it is believed that it should be a feasible target for eradication in much the same way as with the domestic forms of *Triatoma infestans* in the Southern Cone region in South America. There is no doubt that in the past some of the sylvatic *R. prolixus* populations in Venezuela were misidentified *R. robustus* I, and that reports of sylvatic *R. prolixus* from the Amazon region were, in the same way, misidentifications of *R. robustus* II-IV. Moreover, it is the most likely explanation for the observation, in Venezuela, of 'huts long inhabited by men and triatomine-free, although surrounded by "*R. prolixus*" infested palms'. However, it should be considered that at least in areas in Venezuela where true sylvatic *R. prolixus* populations seem to occur re-colonization of domiciles by sylvatic insect populations might be a concern. On the other hand, all four *R. robustus* clades appear to represent entirely sylvatic species, and it is not clear why these populations have been unable to make the transition to domestic environments as *R. prolixus*. Although there are no reports, to date, of *R. robustus* colonizing houses, it can be found occasionally in human habitations, where it flies in from neighboring palms, attracted by light. The epidemiologic significance of these accounts is negligible in comparison with disease transmission mediated by *R. prolixus*, but localized cases are likely to occur in some areas.

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RTE2 - STRAIN DISCRIMINATION OF *EIMERIA* SPP. OF DOMESTIC FOWL: APPLICATION OF RAPD, MITOCHONDRIAL GENOMES AND MICROSATELLITE MARKERS FOR EPIZOOTIOLOGICAL STUDIES

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Coccidiosis of the domestic fowl is a worldwide disease caused by obligatory intracellular protozoa of the genus *Eimeria*. Seven distinct *Eimeria* species are considered pathogenic to chickens: *E. tenella*, *E. acervulina*, *E. maxima*, *E. necatrix*, *E. brunetti*, *E. praecox* and *E. mitis*. Because different species and/or strains can vary in pathogenicity, drug resistance, and other biological parameters, the precise discrimination is important for epizootiological studies as well as for checking the purity of reference and/or vaccine strains. Different molecular markers have been used for species and strain differentiation, including isoenzymes (Shirley, 1975; Johnston & Fernando, 1997), RFLPs (Shirley, 1994), and RAPD (MacPherson & Gajadhar, 1993; Procunier *et al.*, 1993; Shirley & Bumstead, 1994; Johnston & Fernando, 1995). Our group has recently described the development of RAPD assays for the intra- and inter-specific discrimination of *Eimeria* spp. of domestic fowl (Fernandez *et al.*, 2003a). A total of 110 distinct decamer primers generated band profiles specific for each one of the seven species. A subset of 14 oligonucleotides were also tested for the simultaneous differentiation of the seven species, resulting in 11 discriminative primers. The intra-specific discrimination was assessed for five different species, using samples from different geographic sources of three distinct continents. Numerous primers exhibited highly discriminative band profiles containing strain-specific markers, with a higher variability being observed among strains of *E. acervulina* than among *E. tenella* and *E. maxima* strains. RAPD is a powerful technique, but suffers from a poor reproducibility, especially when performed across different laboratories. Due this fact, we decided to convert the RAPD markers into SCARs (sequence-characterized amplified regions), which can be amplified by a pair of specific primers under stringent conditions (Paran & Michelmore, 1993). A total of 138 SCAR markers were developed for the seven *Eimeria* species that infect chickens. A relational database for the SCAR markers was also developed, allowing for SQL-based queries as well as pre-defined searches using a web interface. This database will be made available to the public in a few months at the address http://genoma.fmvz.usp.br/eimeriaScardb/. The SCAR markers were also used for the development of an integrated, cost-effective and simple multiplex PCR assay that permits the simultaneous discrimination of the seven *Eimeria* species in a single-tube assay (Fernandez *et al.*, 2003b).

Once species diagnosis of *Eimeria* spp. was established, we decided to study new approaches for improving strain differentiation. RAPD had already shown some intra-specific variability in several species (Fernandez *et al.*, 2003). However, considering that field samples frequently contain a complex mixture of different species and strains, RAPD may generate overlapping band profiles that are not informative. In order to develop new molecular diagnostic tools for epizootiological studies, we decided to characterize the mitochondrial genomes and look for potential diagnostic targets. In this regard, our group has determined the complete mitochondrial sequences of the seven *Eimeria* species that infect the domestic fowl. In addition, the mitochondrial genomes of 5 distinct strains of *E. tenella* were also sequenced, thus allowing the characterization of the intra-specific variability. These strains were originally isolated in the US, UK and Brazil. Two haplotypes were found, with only two consecutive timines being deleted in strains MC (Brazilian) and Wisconsin (North American), when compared to strains H, TA and Wey (isolated in the UK). This high level of conservation has been already reported for *Plasmodium falciparum* strains isolated from different continents (Conway *et al*. 2000), and may reflect a very recent common origin of the strains. This strikingly high conservation was also observed when the ribosomal ITS1 sequence was determined and compared for these five *E. tenella* strains, revealing no single polymorphism along this region. In view of these results, it became clear that new targets should be pursued before molecular epizootiology studies could be performed among different strains of *Eimeria* spp.

In order to identify microsatellite markers able to detect intra-specific variability, we started an extensive survey using genomic shotgun sequences already available from the *E. tenella* Genome Project in the UK (http://www.sanger.ac.uk/Projects/E_tenella/), as well as cDNA sequences generated in our laboratory for the *Eimeria* ORESTES Project (http://www.lbm.fmvz.usp.br/eimeria/). The sequences were analyzed with the program Tandem Repeats Finder (Benson, 1999) and parsed with a locally developed script (TRFparser). Microsatellite *loci* presenting repetitive clusters of at least seven trinucleotide units, and flanking regions that permitted primer design, were selected. A total of 68 markers (49 for *E. tenella* and 19 for *E. acervulina*) were validated for their ability to generate detectable and species-specific amplicons. From this initial screening, 46 markers (32 for *E. tenella* and 14 for *E. acervulina*) were selected for further tests, using DNA samples from Brazilian, North American and European strains. A partial survey revealed so far 11 polymorphic markers (6 for *E. tenella* and 5 for *E. acervulina*). Futher studies are underway, aiming at the development of at least 10 markers for each one of the most important species: *E. tenella*, *E. acervulina* and *E. maxima*. These markers will be used to check the purity of live vaccine strains, detect unauthorized use of commercial strains, and monitor parasite populations in the field for epizootiological studies.

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RTE3 - PHYLOGENETIC RELATIONSHIPS AMONG *TRYPANOSOMA RANGELI* ISOLATES FROM MAN, WILD MAMMALS AND TRIATOMINES FROM DISTINCT GEOGRAPHIC REGIONS.

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The phylogenetic relationships among trypanosome isolates from man, domestic and wild mammals and their invertebrate vectors are a crucial problem in the evolutionary history of American trypanosomiasis. This study was focused on understanding of the evolutionary relationship and taxonomic position of *T. rangeli*. *T. cruzi* and *T. rangeli* are the only trypanosomes infecting man in the Latin American countries, sharing vertebrate and invertebrate hosts.

Sylvatic mammals described as natural hosts of *T. rangeli* can be infected by the other *Herpetosoma* species, by *T. cruzi*, and by several species of subgenera *Megatrypanum*. Trypanosomes from wild mammals have been identified as a number of different *T. rangeli*-like or allied species on the basis of their resemblance to *T. rangeli* in blood and/or in culture and their different behavior in distinct triatomine species. The prevalence of these trypanosomes in wild mammals is high, and in neotropical monkeys seems to be more prevalent than *T. cruzi.* However, due to the small number of wild isolates studied, the relationships and taxonomic position of trypanosomes related to *T. rangeli* need to be revised since host origin, morphology and triatomine behavior are insufficient taxonomic criteria and the scarce data pointed to misclassification.

Therefore, while evolutionary relationships and taxonomic position of isolates of *T. rangeli* from human and triatomines could be well defined by previous studies (Steven et al., 1999, 2001), trypanosomes from sylvatic mammals classified as *T. rangeli, T. rangeli*-like and allied species were underestimated.

We isolated *T. rangeli* from sylvatic mammals of Brazilian Amazon region, especially from non-human primates and characterized them by comparing

morphology and their behavior in triatomine bugs and mice. The isolates showed to be morphologically indistinguishable and were proven to be *T. rangeli* by detection of metacyclic trypomastigotes in the salivary glands of triatomine bugs. These isolates were also characterized molecularly with the following purposes: a) to evaluate the polymorphism among isolates from wild mammals and from man of the Brazilian Amazon and their genetic relatedness with isolates from other geographic origin; b) to investigate if the populations from sylvatic animals could be differentiate according to their host-species and/or geographic origin; c) to define the relationships of *T. rangeli* with *T. rangeli*-like and allied trypanosomes; d) to identify taxonomic markers.

Genetic polymorphism determined by the randomly amplified polymorphic DNA (RAPD) method revealed that Brazilian *T. rangeli* isolates from the Amazon region can be divided in two groups: Group A, constituted by Brazilian isolates that clustered together with a previously described group originally formed for isolates from Colombia and Venezuela; Group B composed for isolates that differed from those of other geographic regions (from Central America, Southwest South America and Southern Brazil), thus constituting a new group of *T. rangeli* isolates;. Panamanian isolates clustered with other from El Salvador to form another group and one isolate from Southern Brazil did not cluster to any the above-mentioned groups. In this work we demonstrated that the complexity of *T. rangeli* is higher than previously described, with at least 4 distinct genetic groups.

On account of *T. rangeli* unusual combination of stercorarian and salivarian features, producing infective forms in both the posterior and anterior stations of vector, its systematic position has been a subject of controversy. Due to complex genetic variability and considering that, besides selection for human and insect isolates, previous studies included few isolates and lacked members of all genetic groups, we do not have yet sound data to define the evolutionary relationships among *T. rangeli* isolates as well as the relatedness between this species and allied trypanosomes.

The study of a large number of isolates from distinct host species and geographic origin is indispensable to define the taxonomic position and to evaluate the factors determining the segregation of *T. rangeli* in lineages, their evolutionary relationships and thus, their taxonomic status. With this purpose, it was inferred phylogenetic relationships among 36 isolates of *T. rangeli, T. rangeli*-like or allied species (*T. saimiri*, *T. preguici*, *T. leeuwenhoeki)* and isolates previously classified as *Megatrypanum* species which revealed to be *T. rangeli* (*T. minasense* and *T. legeri*) by comparison of small-subunit of ribosomal RNA (SSUrRNA) gene sequences. *T. rangeli, T. rangeli*-like and related species clustered together and this cluster was more closely related to *T. cruzi* than to *T. lewisi* or to *T. brucei*.

Taken together, biological and molecular features have now been gathered to demonstrate that *T. rangeli* isolates from distinct host and geographic origin and allied species can be tightly clustered together into the subgenus *Tejeraia,* within the Stercoraria. Moreover, the phylogenetic trees produced strongly supported that the subgenus *Herpetosoma* is polyphyletic, and can be divided in two monophyletic lineages representing by *T. rangeli* or *T. lewisi.* Based on phylogenetic analysis and considering the differences on morphology, behavior in vertebrate and invertebrate hosts and epidemiology we propose the maintenance of *T. lewisi* as the type-species of the subgenus *Herpetosoma* and the definitive validation of the subgenus *Tejeraia,* with *T. rangeli* as its type-species.

Despite the close relationships among isolates of *T. rangeli* and allied species, the branching pattern of the phylogenetic tree based on SSU rRNA sequences revealed four well supported phylogenetic groups. Moreover, polymorphism of the more divergent sequences of the internal transcribed spacer (ITS) of ribosomal gene also permitted the distribution of the trypanosomes into the same phylogenetic groups previously defined by SSUrRNA and RAPD analysis.

In this study we also compared the spliced-leader (SL) gene sequences of *T. rangeli* isolates and allied species to evaluate the degree of genetic relatedness among isolates of *T. rangeli*, *T. rangeli*-like and allied species from distinct host species and geographic regions. Both the length and sequence of the SL gene repeat of isolates of *T. rangeli*, *T. rangeli*-like and allied species showed high similarity whereas differed remarkably from other trypanosome species within both intron and intergenic spacer regions. However, the alignment of SL sequences showed consistent levels of genetic diversity on both intron and intergenic spacer. The dendogram based on SL sequences clearly separated these organisms into four major groups. Groups defined by SL were identical to those generated using RAPD and ribosomal markers, again confirming the high complexity of *T. rangeli.* A *T. rangeli*-specific diagnostic PCR assay based on SL intergenic sequences was developed to define sequences suitable for simultaneous diagnosis of *T. rangeli* isolates from Central to South America and to type the isolates in groups according to the size of the amplified fragments.

Besides biological diversity regarding behavior in triatomine bugs, several biochemical and molecular features have demonstrated polymorphism among *T. rangeli* isolates, segregating the isolates into two groups: isoenzymes and RAPD profiles (Steindel et al., 1994); kDNA minicircles (Vallejo et al., 1994; Vallejo et al., 2002) and mini-exon sequences (Grisard et al., 1999; Vallejo et al., 2003).

In this study, grouping using SSU and ITS ribosomal gene, RAPD and spliced-leader gene markers, besides confirming these two previously described groups, are all in agreement to the distribution of *T. rangeli* in at least four groups of more genetically related isolates. Thus, molecular characterization of a large number of *T. rangeli* isolates using several sequences and approaches indicated that this species is a very complex taxon, constituted by different populations presenting particular genetic characteristics. Taken together, there are enough data to assert the separation of *T. rangeli* isolates in highly consistent and homogeneous phylogenetic groups.

The same grouping of *T. rangeli* isolates suggested clonal evolution due to non-random association of independent markers. Populations from different host species constitute all groups of *T. rangeli,* thus discarding any association of groups and vertebrate species. Moreover, although there are some geographical distance determination of segregation patterns, populations from the same or neighbor regions can be typed in more than one group, indicating that other factors are also determining the segregation of these organisms.

Several studies on triatomine behavior revealed high restriction of some *T. rangeli* isolates to their local vector species. Analysis based on kDNA and mini-exon gene supported either clonal evolution or speciation of *T. rangeli* populations in their triatomine vectors (Valejjo et al., 2003). We are currently investigating if differences on isolates from regions of Brazilian Amazon not separated by significant geographical barriers can be due to specific adaptation to particular triatomine species. More isolates of distinct hosts and geographical regions and new molecular markers must be investigated to make definitive statements concerning the determinant factors of this segregation as well as some taxonomic status for each group.

This is the first study that assesses the genetic relationship of large number of isolates from wild mammals, especially from non-human primates, demonstrating that isolates from sylvatic mammals and man are highly genetically related, confirming the lack of host-restriction of *T. rangeli* and suggesting that the same parasite can circulates among sylvatic mammals, humans and triatomines.

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RTE4 - THE USE OF MICROSSATELITE DNA MARKERS IN MOLECULAR EPIDEMIOLOGY OF MALARIA

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Polymorphisms in genes encoding antigens and targets of anti-malarial drugs have been largely used to examine the population structure of *Plasmodium falciparum.* However, natural selection may be more important than local patterns of transmission dynamics and population structure of malaria parasites in determining the diversity and differentiation of these non-neutral loci among

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populations. Putatively neutral markers (polymorphisms located in non-coding DNA sequences that are not under selective pressure), such as most hypervariable microsatellites (arrays of short repeat DNA sequences), provide an alternative to circumvent these shortcomings[1]. Here we present examples of the use of microsatellite markers to investigate the temporal and spatial patterns of genetic diversity and differentiation of world-wide *P. falciparum* populations[2], the levels of genetic relatedness of isolates that share phenotypes of interest, such as the ability of causing severe malaria[3] or the expression of common antigenic variants of merozoite surface proteins[4], and the origin and geographical spread of resistance to the anti-malarial drug chloroquine.

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RTIM1 - MUCOSAL AND SYSTEMIC IMMUNE PROTECTION AGAINST EXPERIMENTAL CHAGAS' DISEASE.

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Trypanosoma cruzi is transmitted to humans and other mammalian reservoirs by mucosal, cutaneous and systemic routes. An ideal vaccine for Chagas' Disease would induce immunity protective against all modes of transmission possible with this protozoan parasite. We have developed murine models of conjunctival, oral, contaminative cutaneous, and parenteral *T. cruzi* infection, and are actively studying the regional molecular immune requirements for protection in the relevant mucosal and systemic lymphoid tissues. After conjunctival and oral challenges *T. cruzi* invades and replicates locally within the mucosal epithelia, then spreads to the relevant draining lymph nodes, and finally by 2 weeks post-challenge disseminates throughout the blood and lymph. Similarly, *T. cruzi* replicates first within local cutaneous tissues at the site of contaminative cutaneous challenge, prior to the development of patent parasitemia. Therefore, during the first 2 weeks after mucosal and cutaneous challenges, real-time PCR and quantitative culture techniques can be used to estimate the levels of regional replication at the initial sites of invasion, and these techniques can be used to study regional immunity separately from systemic immunity in control and immunized mice. In these murine models we have evaluated the importance of Type 1 immunity (associated with IFN-γ, IL-2, TNF-α & optimal CTL responses) and Type 2 immunity (associated with IL-4, IL-5 and IL-10 responses) for mucosal and systemic protection. Both cytokine biased vaccination approaches and immune knockout studies conclusively indicate that Type 1 responses provide optimal mucosal and systemic *T. cruzi* protective immunity. We have developed molecular vaccines expressing cruzipain and trans-sialidase and demonstrate that these vaccines can induce both mucosal and systemic protection. The importance of $CD4^+$ T cells, $CD8^+$ T cells and B cells in cruzipain and trans-sialidase immunity are being evaluated in knockout mice and adoptive transfer experiments. In preliminary experiments, all 3 populations of lymphocytes are important for protection induced by intranasal vaccinations with recombinant trans-sialidase and CpG adjuvant. The B cell knockout studies further suggest that B cells provide important antigen presenting functions for the induction of T cell responses. In addition, we are currently studying the possibility that prime/boosting regimens using combinations of recombinant soluble proteins mixed with CpG, DNA vaccines and live vaccine vectors expressing cruzipain and/or transialidase, can induce sterile mucosal and systemic protection. Finally, we are investigating the integrin and chemokine receptor molecules involved in trafficking of protective T cells to mucosal, cutaneous and systemic tissues. We hypothesize that mucosal prime/boosting immunization strategies that focus mucosal and systemic vaccine responses on key protective antigens can be highly protective against Chagas' disease by: 1) preventing initial *T. cruzi* infection and 2) limiting the burden of parasite replication in individuals that become chronically infected after *T. cruzi* challenge.

RTIM2 - DIFFERENTIAL PRODUCTION OF INFLAMMATORY CYTOKINES IN CCC: MODULATION OF GENE AND PROTEIN EXPRESSION IN CCC HEART LESIONS

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In spite of the effective control of vectorial transmission of *T. cruzi* infection, there are still millions of patients already infected with the parasite, from which up to one third will develop life-threatening Chronic Chagas disease cardiomyopathy (CCC). No treatment modality has slowed the progression to end-stage CCC in accurately controlled clinical trials, the likely cause of its worse prognosis. Susceptibility factors leading 30% of infected patients to develop CCC are largely unknown. The identification of pathogenesis checkpoints is thus very important to allow focus on new therapeutic targets related to disease progression. Our group and others have shown that T cells infiltrating the heart of CCC patients predominantly produce Interferon-gamma (IFN-gamma) and TNF-alpha in the absence of IL-4 and limited production of IL-10 (1, 2, 3), suggesting that heart damage may be secondary to inflammatory cytokines. Chronic *T. cruzi* infection is associated to significanly increased levels of plasma TNF-alpha (4), as well as *T. cruzi* B13 antigen/PHA-driven interferon gamma production by peripheral blood mononuclear cells (PBMC) (1). Since we have previously observed that live *T. cruzi* trypomastigotes induce IL-12 and TNF-alpha production by human PBMC, this suggests that the potent prostimulatory activity shifts the cytokine profile of *T. cruzi*-infected patients. Furthermore, the frequency of IFN-gamma-producing PBMC is increased in CCC patients as compared to asymptomatic-"indeterminate" (ASY) patients or normal controls, suggesting that CCC patients develop an increased number of T1-type T cells (1,5), possibly as a consequence of differential response to the chronic proinflammatory stimulus by *T. cruzi*.

We have addressed the question of a differential response of CCC, ASY patients or N individuals by analyzing the production of IFN,gamma, TNF-alpha and IL-10 by PBMC after stimulus by TLR-4 ligand or PHA. Although the three cytokines were stimulated by either treatment, we observed that CCC PBMC under LPS stimulus produced significantly higher IFN-gamma and TNF-alpha, and significantly lower IL-10, than ASY or N controls. On the other hand, the cytokine production profile of CCC PBMC under PHA stimulus was similar to that of ASY and N. This suggests that the TLR-4 pathway, and possibly the monocyte, are the targets for the differential response to *T.cruzi* proinflammatory stimulus. That TNF-alpha plays a damaging role in CCC was confirmed by the observation that among end-stage CCC patients, those bearing the TNF2 and TNFa2 alleles of TNF polymorphic sites, associated with high TNF production, have a significantly shorter survival (unpublished data).

In order to assess the tissue response to inflammation at the molecular level, we analyzed gene expression in 5 heart tissue samples from end-stage CCC and idiopathic dilated cardiomyopahy heart explants and heart donors, using a 10,386

element cDNA microarray, the Cardiochip. We found that the cardiac hypertrophy signature was upregulated in both CCC and DCM hart tissue. However, immune-response related genes, and among them, IFN-gamma-induced genes were prominently induced only in CCC patients. Quantitative Real time PCR with selected primers confirmed the array data, and additional primers showed showed that IFN-gamma-induced chemokines MIG, IP10, and their receptor MCP-1 are significantly upregulated only in CCC heart samples.

Using proteomic analysis (2D gel electrophoresis and peptide mass fingerprinting), we compared the protein profile in CCC and DCM hearts. Preliminary analysis of the 200+ protein spots in each gel identified the most prominently downregulated protein in CCC hearts as mitochondrial creatine kinase. Mitochondrial creatine kinase mRNA was also downmodulated in CCC heart explants. Since mitochondrial creatine kinase plays a key role in the generation of cytoplasmic ATP from mitochondrial sources, this might be one of the mechanisms underlying the worse prognosis in Chagas disease. Given the fact that genes for other mitochondrial creatine kinase isoforms are repressed by IFN-gamma, it can be hypothesized that IFN-gamma induces harmful modulation of gene expression in the myocardial cells themselves, in a novel pathogenic mechanism.

Thus, our data allow us to hypothesize that after *T. cruzi* infection, the extent of the T1-type cytokine shift, differential response to proinflammatory/TLR stimulus, and cytokine genetic polymorphisms may contribute to the extent of inflammation-mediated heart tissue damage at the level of myocardial gene and protein expression in CCC.

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RTIM3 - THE INVOLVEMENT OF AUTOIMMUNITY AGAINST RETINAL ANTIGENS IN DETERMINING DISEASE SEVERITY IN TOXOPLASMOSIS[1].

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Disease intensity in ocular toxoplasmosis varies greatly between patients. Autoimmunity has been suggested as a possible component to retinal destruction. We decided to study the cellular immune response to several ocular antigens in patients with ocular toxoplasmosis with the purpose of associating it to disease severity. Patients with mild disease responded to one or more retinal antigens with a significantly higher frequency than patients with severe disease. IL-2 and IFN-γ synthesis followed the same pattern of the lymphoproliferative response; IL-4 and IL-5 were seldom detected. Interestingly, IL-10 and IL-12 p40, but not IL-12 p70, were synthesized by seronegative patients in response to retinal antigens. Individuals with ocular pathology did not secrete significant levels of either one of these cytokines. Our results suggest that although the presence of an immune response towards autoantigens is not protective against the development of ocular lesions by the *Toxoplasma gondii*, it may protect against the development of severe disease.

Keywords: Autoimmunity, Parasitic-Protozoan, Cellular Proliferation

RTIM4 - INDUCTION OF HIGH LEVELS OF IL-12 P40 BY METACYCLIC LEISHMANIA IS RELATED TO AN IMMATURE STAGE OF MACROPHAGE.

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Production of IL-12 is an important indicator of the macrophage's ability to regulate immune responses and it is accepted that the maintenance of immunity against *Leishmania* is dependent on the continuous production of this cytokine. Although IL-12 is produced by draining lymph node cells 24-48 h after infection of the mouse with metacyclic *Leishmania* (Vieira *et al.*, 1994; Oliveira *et al.*, 2000), *in vitro* infection of murine macrophages with metacyclic *Leishmania* fails to stimulate IL-12 production and in addition can inhibit its synthesis (Reiner *et al.*, 1994; Vieira *et al.*, 1994; Carrera *et al.*, 1996; Oliveira *et al.*, 2000). It is suggested that initial production of IL-12 by host cells stimulated with infective parasites comes from dendritic cells, since IL-12 could be detected in supernatants from murine dendritic cells cultures when stimulated for 24 h with *Leishmania* promastigotes (Konecny *et al.*, 1999). However, the levels of IL-12p40 detected in cultures of purified murine dendritic cells stimulated with *L. major* promastigotes (Konecny *et al.*, 1999) are much lower than the levels of this cytokine as observed by other authors using cultures from whole lymph node cells (Vieira *et al.*, 1994; Scharton-Kersten *et al.*, 1995; Oliveira *et al.*, 2000). This implies that other cell populations could collaborate with dendritic cells to increase metacyclic induced IL-12 production *in vivo*.

In this study, we investigated the IL-12 production by myeloid cells at several developmental stages when stimulated with different species of *Leishmania*. To this end, bone-marrow cells were cultured for 4-6 days in vitro in presence of M-CSF, GM-CSF or IL-3. Density separation with Percoll yielded populations that were enriched in cells at different maturation stages. Invariably, only cells banding at the 40-50% Percoll interface produced large amounts of IL-12p40 when stimulated with live *Leishmania*, whereas only low levels of IL-12p70 were produced. Heat killed *Leishmania* or total *Leishmania* extract were unable to stimulate IL-12p40. The high-IL-12-producing cells could be derived from C3H, C57BL/6 or BALB/c mouse strains and similar amounts of IL-12 p40 were produced. By employing quantitative analysis of monocyte/macrophage lineage differentiation markers associated to positive and negative selection of cells carrying these markers it was possible to conclude that the high IL-12p40-producing cells were immature macrophages, since these cells had already lost cell surface markers characteristic of immature myeloid cells, such as CD31/ER-MP-12, Ly-6C/ ER-MP20 and ER-MP58, but had acquired the marker ER-HR3, characteristic of mature macrophages. The IL-12 producing-cells also expressed low levels of scavenger receptor, likewise a marker of mature macrophages and may or may not express CD11b/Mac-1, also increasingly expressed by mature cells.(Chan *et al.*, 1998; Leenen *et al.*, 1998; McKnight and Gordon, 1998). By flow cytometry, we were unable to detect any labeling of these cells for the classical dendritic cell markers 33D1 or CD11c.

Our work shows that promastigote forms of *Leishmania* are able to induce the production of high amounts of IL-12p40 by immature cells derived with M-CSF, IL-3 or GM-CSF. However, this high production of IL-12p40 is not related to a high production of the active cytokine IL-12p70 *in vitro*. IL-12 is one of the main cytokines that drives the immune response to a resistant phenotype in leishmaniasis and in several other diseases but, on the other hand, IL-12p40 can also exert a negative regulatory activity on T cell activation. Besides *Leishmania*,

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we have recently shown that other microbial constituents like bacterial LPS, can also stimulate large amounts of IL-12 p40 by immature macrophages (Oliveira *et al*., 2003). These findings may have important implications to understand the maintenance of IL-12 synthesis in the draining lymph nodes during *Leishmania* infection as well as the regulation of T cell activation and differentiation in infections by Leishmania and microorganisms that have LPS. The ability to control parasitism as well as the immunopathology of the lesions may be influenced by the stimulatory or regulatory activity exerted by IL-12 synthesized by immature macrophages that migrate from the blood to the lesion and/or lymph nodes.

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RTMB1 - SPLICED LEADER RNA PROCESSING IN KINETOPLASTID PROTOZOA

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The spliced leader (SL) RNA is the common substrate for trans-splicing of every mRNA transcribed in the nucleus of a kinetoplastid. Acquisition of the 39-nt SL confers stability and translatability on to mRNAs in the form of a unique 5’-end structure referred to as cap 4. The cap 4 consists of four 2’-*O*-methylations of the first four nucleotides (AACU) and base methylations at positions 1 and 4. A pseudouridine is also formed at position U_{28}. The intron portion of SL RNA contains two stem-loop structures flanking a Sm-binding site typical of small nuclear (sn) RNAs associated with splicing. Thus, the biogenesis of the SL RNA substrate represents a potential target that distinguishes it from host biology. We have employed transfection and/or RNA interference (RNAi) techniques to study the steps and associated components in SL RNA maturation and function in *Leishmania tarentolae* and *Trypanosoma brucei*. Our current working model is as follows: During transcription by RNA polymerase II, the SL RNA receives a 5’-m^7G cap (cap 0). Transcription termination is staggered in a T tract lying adjacent to the gene sequence of the mature transcript. The primary transcript contains the m^7G cap and additional nucleotides at the 3’ end. The nascent SL RNA is likely recognized by the nuclear cap binding complex (CBP20/CBP80) and then exported from the nucleus via the Exportin 1 pathway. Blockage of SL RNA nuclear egress can be affected by the use of cytotoxin Leptomycin B, which specifically inhibits exportin 1 function; cells treated with LMB retained SL RNA in the nucleus and accumulated 5’-cap 0 and 3’-extended SL RNA. The SL can receive the first and possibly the second of the cap 4 methylations at positions 1 and 2 independent of Sm-complex interaction. SL RNA associates with Sm proteins in the cytosol, permitting progression of cap 4 methylation and initiation of 3’ end trimming. Mutagenesis of the Sm-binding site on the SL RNA or knock-down of the SmD1 protein, a component of the Sm complex, by RNAi yielded similar phenotypes including incomplete cap 4 formation, loss of 3’ processing and general over-accumulation of SL RNA. Multiple enzymes are likely to be involved in cap 4 methylation, and a minimum of two ribonucleases are indicated in the 3’ processing of SL RNA. Rudimentary formation of cap 4 and/or association with the Sm complex likely provide the signal for nuclear import. We have identified the final nuclease, dubbed SNIP for snRNA incomplete processing, responsible for the trimming of the final 3’ nucleotides of SL RNA in the nucleus, as well as for 3’ trimming of the U2 and U4 snRNAs and 5S rRNA. Currently we are examining the possible role of exosome components in the initial 3’ processing of SL RNA. The specific cap 4 methylations and pseudouridylation have yet to be placed within the intercellular maturation pathway. A role for either cap 4 or the primary SL sequence is implied in translation, as SL mutants deficient in cap 4 are not efficiently loaded onto polysomes.

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RTMB2 - TELOMERES AND TELOMERIC PROTEINS OF *LEISHMANIA* SPP.

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The ends of eukaryotic linear chromosomes are capped by a nucleoprotein structure known as telomeres. Telomeres ensure the complete DNA replication, prevent end to end fusion and help to differentiate the chromosome terminus from double strand breaks, being, thus, essential to maintain the integrity of the genome.

We are attempting to understand the telomere biology of *Leishmania*, the causative agent of leishmaniasis. Our interests are focuses not only in the origin and features of these structures in a primitive organism, but also in the importance of telomeres in parasite life span.

Leishmania spp. telomeres are composed by the conserved TTAGGG repeated sequence, which is present in high copy number in each parasite chromosome. Different experimental evidence has shown that in *L. amazonensis* non synchronized cells, the length of the terminal restriction fragment (TRF), has a mean size of approximately 7 Kb, from which 3 Kb comprises the telomeric sequences. Different from other species, *L. (L.) amazonensis* LCTAS (*Leishmania* Conserved Telomere-Associated Sequences) are composed mainly by tandem arrays of CSB2 blocks, flanked by *Hae*III restriction sites, and appear in low copy number per chromosome ends. In addition, CSB2 blocks are highly sensitive to *Bal*31 digestion, indicating that besides of being interspersed by telomeric repeats, they are probably located

immediately adjacent to terminus of the chromosomes. The characterization of telomere-derived clones are in progress and may shed new lights about telomere organization in *Leishmania*.

Telomerase is responsible for replicates parasite telomeres and we are analyzing the *in vitro* enzyme catalysis by using extracts that were highly purified in 2'-*O*-methyl affinity chromatography columns. Preliminary results show that *L. amazonensis* telomerase activity is moderately processive, is inhibited by RNAseA and has elongation properties characteristic of other telomerases. By database searching analysis we were able to identify a *L. (L.) major* sequence that shows homology to the conserved motifs that are hallmarks of the catalytic region of all identified telomerase protein genes. Similar sequences were cloned from *L. (L.) amazonensis*, *L. (L.) braziliensis* and *L. (L.) major*, using a PCR-based strategy. Since none of the PCR products are full length, we are currently performing RACE-PCR to obtain the NH2 - and C-terminal sequences. Truncation and overexpression of one of these genes will probably indicate if they encode the reverse transcriptase catalytic gene of *Leishmania*.

Apart from telomerase, proteins that compose the parasite telomeric chromatin are also being biochemical and genetically studied. Three protein-DNA complexes (LaTG1-LaTG3) that specifically associate to the G-rich telomeric strand were characterized in nuclear and S100 extracts of *L. (L.) amazonensis*. The protein components of two complexes were identified as being homologues of the conserved subunit 1 of replication protein A (RPA-1) and the trypanosomatid RNA-binding protein RBP38, respectively. Moreover, genetic selection of double-stranded telomeric proteins using yeast one-hybrid system identified novel parasite proteins that share no homologies with proteins described in other eukaryotes.

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RTMB3 - *PLASMODIUM* TELOMERES: A PATHOGENS' PERSPECTIVE

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The nuclear organisation of the *Plasmodium falciparum* genome determines many important biological processes. Compartmentalization highlight different processes that are involved in parasites specific evasion processes such as antigenic variation. For example, clustering of chromosome ends enhances ectopic gene conversion events between telomeric gene families such as *var* genes (Freitas-Junior et al., Nature. 2000. 407:1018-22). The epigenetic mechanisms that control mutually exclusive *var* gene expression at the surface of infected erythrocytes, a process known as antigenic variation, are still unknown. We investigated whether changes in the nuclear localization or the chromatin status of expressed and silenced telomeric *var* genes are essential components of this control mechanism. Although the factors involved in the reversible gene silencing remain unknown, we have identified *P. falciparum* genes orthologous to most *S. cerevisiae* genes involved in the epigenetic regulation of telomere associated genes such as Sir1-4 and Ku binding protein (Scherf et al., Curr Opin Microbiol. 2001. 4: 409-14). Antibodies raised against these antibodies indicate that these proteins are concentrated at the telomere foci and in a second compartment, the nucleolus. Chromatin precipitation (Chips) of these antibodies specifically enriched for telomere and telomere associated DNA elements, pointing to Sir proteins as critical elements in silencing of telomeric var genes. This is supported by FISH studies that demonstrate that telomeres loci appear much more close together than comparable loci located in chromosome central regions.

RTMB4 - SATELLITE DNA ORGANIZATION IN *TRYPANOSOMA CRUZI* STRAINS

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Satellite DNAs are repeated non-transcribed sequences present in eukaryotic systems and in a typical chromosome satellites are organized in megabases tandemly localized at heterochromatic regions of the genome. Despite their abundance in all eukaryotes, they are rarely described in early diverging eukaryotes, such as protozoa. Exceptions are the satellite sequences found in trypanosomes, in which they represent the most abundant repetitive sequence present in about 10^5 copies in the genome. We studied satellite DNA organization in representative strains of the two major phylogenetic groups of T. cruzi [*T. cruzi* I (Silivio X10 cl1) and *T. cruzi* II (Y)] and we also investigated the satellite DNA organization in CL Brener, the reference organism of *T. cruzi* genome project. We found that despite the amounts of these repeats vary among strains, they are grouped in clusters comprising about 30 + 10 kb in different chromosomes. These clusters are constrained in specific regions of chromosomes as evidenced by fluorescence hybridization assay of spread DNA fibers. These data point to a similar way of satellite organization in these three strains, suggesting that they have conserved an important structural role in *T. cruzi* chromosomes. Sequence analysis of several repeat units of satellite DNA of the three strains revealed that Cl Brener satellite DNA has two sets of sequences: one represented by 12 sequences (12/45; 26.6%) that clusters with Silvio (*T. cruzi* I) satellites and the other, represented by 33 sequences (33/45; 73.3%), that clusters with Y strain (*T. cruzi* II) sequences. The divergence between Silvio and Y strains satellite sequences is significant (10% sequence divergence on average). Sequences of the same strain have 2% sequence divergence on average. Moreover, satellite sequences derived from the same CL Brener chromosome cluster together and have > 95% sequence identity. This indicates that *T. cruzi* satellite sequences are chromosome specific. The phylogeny also reveals that CL Brener strain contains some chromosomes derived from a *T. cruzi I* and other chromosomes derived from a *T. cruzi II*-like ancestors. We conclude that the genome of CL Brener contains chromosome derived from *T. cruzi* I and *T. cruzi* II parental lines or ancestors. This suggests that hybridization, recombination and genetic exchange occur in *T. cruzi* natural populations.

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RTVE1 - IMMUNE RESPONSE TO VECTOR SALIVARY PROTEINS: FROM SPITOMES TO FUNCTIONAL GENOMICS.

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Blood feeding arthropod's saliva is a cocktail of biologically active components that affects the host hemostatic, inflammatory and immune system. These components modify the physiology of the host at the site of the bite; pathogens take advantage of this change and become more infective. Vertebrate immune response to arthropods feeding or bites (possible to salivary proteins) results in protection against certain vector borne diseases, therefore, salivary proteins are attractive targets to control vector borne disease.

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We have modified our approach to isolate and test vector salivary proteins, we moved from one protein-one gene to high throughput approaches based on massive cDNA sequencing, proteomics and computational biology to isolate and identify a large number of proteins and genes and to select potential vaccine candidates from the salivary glands of vectors of disease.

The next challenge was how to test the large number of vaccines candidates identified with this high throughput approach. For this we developed a high-throughput DNA vaccine construction protocol, which is accelerating the discovery of salivary molecules, which can induce an immune response in the vertebrate host. We have identify a number of salivary proteins from the salivary glands of the sand fly *Phlebotomus papatasi*, *Phlebotomus ariasi* and *Lutzomyia longipalpis* which induce 1) a strong cellular response in the skin of the host, 2) a strong antibody response and 3) produce both responses. We are presently studying these immune responses and their potential role as vaccine candidates to control *Leishmania* infection.

RTVE2 - OOGENESIS IN TRIATOMINES

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As in many other insects, in triatomines oogenesis occurs as a consequence of a very complex array of cellular and metabolic events controlled by hormones, that ends up in the formation of an oocyte cell. Following fertilization the egg will develop and give rise to a new insect. To build up an oocyte, the ovary requires substrates that comes from the digestion of food, that in the case of triatomines is the blood of a vertebrate. Following digestion of the blood, proteins, carbohydrates and lipids are transported to the ovary where they are delivered and used to build up a mature oocyte, ready to be fertilized. In order to reach this point of development, several tissues are coordinated to produce proteins, or lipoglicoproteins and enzymes, to be sent to the oocytes where they are stored and used by the new embryo. Among these proteins are vitellogenins (synthesyzed by fat body), lipophorins (lipid carrier), non-vitellin proteins such as enzymes (phosphatases, kinases and proteases), RHBP (*Rhodnius* HemeBinding Protein); RCBP (*Rhodnius* Calcium-Binding Protein) that besides aminoacids carries also heme and calcium to be used by the new embryo. In the hemolymph of *Rhodnius prolixus*, vitellogenin that carries aminoacids, lipids, phosphate and carbohydrates is found as a homogeneous population. Once internalized by receptor-mediated endocytosis, vitellogenin, that now is called vitellin to differentiate it from vitellogenin, appears as a heterogenous population inside the oocyte. Three different populations can be found and they were named VT1, VT2 and VT3 following their order of elution in a DEAE chromatography (Toyopearl). A close analysis of each population revealed that only one population of VT came from vitellogenin from the hemolymph and correspond to VT3 as a result of post-endocytic processing. The group of vitellin named VT1 is synthesyzed by follicle cells especially at the end of vitellogesis, close to the period of chorion deposition. Although similar to other vitellins, VT1 contains different markers compared with other vitellins. On the other hand, the origin of VT2 is not clear, but we now have evidences that vitellin can also be produced by trophocytes at the tropharium. Antibody against vitellin clearly shows that trophocytes contain similar molecules. Besides that, isolated tropharium, incubated in culture medium, synthesize and secrete molecules similar to vitellin. Polypeptides of the same size of vitellin are produced and recognized by antibody against vitellin, suggesting the synthesis “de novo”of vitellin by the tropharium.

Evidences will be presented that VT1 is localized at the outer layer of oocyte suggesting that this population of VT can be the first group of vitellins to be used by the embryo, because the embryo development starts by the nucleous division followed by cellularization exactly at this outer layer of oocyte, and possibly the substrates used for cellularization comes from VT1.

Insect eggs are composed of vitellins and non-vitellins proteins and the role of these different proteins for embryo development will be discussed.

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RTVE3 - TRIATOMINES FEEDING BEHAVIOUR: ELECTROMYOGRAM OF CIBARIAL PUMP AND INTRAVITAL MICROSCOPY

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Triatomines are solenophagic arthropods, obtaining their bloodmeals directly from the blood vessels (venules or arterioles) of their vertebrate hosts. After piercing the host skin, a probing period characterised by rapid whip-like intradermal movements of the maxillae can be observed. Then, when a suitable vessel is found, probing ceases and the bugs engorge on the host blood (Lavoipierre *et al.*, 1959). Ingestion of blood through the food canal is aided by the cibarial pump, a structure whose movements are regulated by a complex of strong muscles occupying much of the insect’s head (Bennett-Clark, 1963). Conservative calculations suggest that the cibarial pump of *Rhodnius* is able to generate negative pressures of 1–2 atm during feeding. Parameters such as negative pressures produced by cibarial pump, dimensions of insect food canal, viscosity, size of host red blood cells and its capacity of deformation can influence ingestion rate of blood-sucking insects (Kingsolver and Daniel, 1995). The electromyogram of cibarial pump permits monitors the feeding process of triatomines (Guarneri *et al.*, 2000, 2003; Sant’Anna *et al.*, 2001). Using this technique, we compared probing time, non-ingestive time, cibarial pump frequency and volume ingested per each pump contraction, as well as how those factors can be influenced when the insects were fed on different hosts. These studies with fifth instar nymphs have shown that the volume ingested by each cibarial pump contraction and maximum frequency obtained using the artificial feeder are related to intrinsic mechanical characteristics of the insect feeding apparatus. However, probing time, the modulation of cibarial pump frequency on live hosts and interruptions during the engorgement phase may be related to salivary function. Thus, mechanical features and salivary function appear to act together, influencing the total ingestion rate. In the experiments carried out using mice, probing times and interruptions tended to be more prolonged while cibarial pump frequency is lower during the meal. This reinforces the idea that feeding on mice is more difficult than on pigeons, requiring more contact time to obtain the similar quantity of blood. Intravital microscopy using *Rhodnius prolixus* saliva labeled with fluorochrome permits the study of salivation rhythm. We observed that the saliva is continuously pumping during all feeding process. This kind of study has been permitting a better comprehension of triatomine-host interaction.

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RTVE4 - INHIBITION OF THE COMPLEMENT SYSTEM BY SALIVA OF HAEMATOPHAGOUS INSECTS: ITS PHYSIOLOGICAL ROLE AND PROBABLE IMPORTANCE FOR PARASITE TRANSMISSION

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The saliva of haematophagous insects has a series of pharmacological activities that interfere with some physiological processes of the vertebrate hosts, thus favouring blood feeding. An inhibitory effect on the complement system was observed in salivary extracts obtained from various haematophagous insects. The inhibition of the classical pathway was measured using sensitised sheep erytrocytes and the alternative pathway, using non sensitised rabbit erytrocytes in an haemolytic assay. When assayed for complement inhibition, saliva from *Lu. longipalpis* was capable of inhibiting both the classical and alternative pathways, while that from *Lu. migonei* acted only on the former. Other haematophagous species were screened for inhibition of the classical pathway. The triatomine bugs *Panstrongylus megistus*, *Triatoma brasiliensis* and *Rhodnius prolixus* were able to inhibit the classical pathway whereas the mosquito *Aedes aegyti* and flea *Ctenocephalides felis* were not. The activity of *Lu. longipalpis* saliva against the classical pathway was partially characterised. The inhibitor is a protein of M.W. 10.000 - 30.000 Da which is very resistant to denaturation by heat. The physiological role of the inhibitory protein is probably to protect the insect gut against lysis by the complement system The anti-complement activity would be very effective in protecting *Leishmania* promastigotes just after their introduction into the skin of a vertebrate host via the bite of a sandfly. Use of the inhibitor molecule is thus a promising component of a vaccine to target salivary immunomodulators.

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