

### BC1 - THE MEDIAN BODY OF *GIARDIA LAMBLIA*: ULTRASTRUCTURE BY HIGH RESOLUTION SCANNING ELECTRON MICROSCOPY

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*Giardia lamblia* is an intestinal parasite of several mammals. It is a parasitic protozoan that infects thousands of people all over the world, causing a disease known as giardiasis. *Giardia* possesses several cytoskeletal structures composed of microtubules, such as the disc, median body, axonemes of eight flagella and the funis. The median body is formed by an irregular set of microtubules situated dorsally to the caudal axonemes, resembling a comma in a face.

In the present study we have used a new technique that allows the removal of the plasma membrane using detergents and observation of cytoskeletal structures by either conventional scanning electron microscopy or field emission scanning electron microscopy (FESEM). Cells were adhered to poly-L-lysine-coated glass coverslips and then treated with the permeabilization buffer (0.5% Nonidet-40 P-40, 0.1M Pipes, 1mM MgSO<sub>4</sub>, 2 mM glycerol, 2 mM EGTA, 1 mM PMSF (phenylmethylsulfonyl fluoride), 0.5% Triton X-100) for different times (10 min-2 h). The cells were washed, fixed in 2.5% glutaraldehyde, post-fixed 1% OsO<sub>4</sub>, dehydrated, critical point dried and coated with gold. The samples were examined in a Jeol JSM-6340F Field Emission or JSM-5800 Scanning Electron Microscope. Upon these procedures the median body was better visualized. When the plasma membrane was completely extracted the median body was not visualized suggesting the connection between these two cells structures. FESEM revealed that the median body was constituted by several small fascicles formed by microtubules forming larger bundles. The bundle number was variable as well the microtubules number found in each fascicle. There was a variation in number, disposition and location of the median body fascicles in different organisms found in the same culture preparation. Each fascicle was formed by parallel microtubules with different lengths, but the different fascicles were not parallel. Some fascicles were connected, leading a misunderstanding by light microscopy of the presence of one or two median bodies. In the caudal axonemes region a thicker median body bundle is seen. In conclusion, there are not two median bodies, and they were present in about 70% of the cells population, with connections with the ventral disc.

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### BC2 - MYCOPLASMA OBSERVATIONS IN *TRICHOMONAS VAGINALIS*: THE ENEMY WITHIN.

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The protist *Trichomonas vaginalis* is a well described early eukaryote and its infection is the most prevalent nonviral sexually transmitted disease worldwide. In recent studies it has been proposed that Trichomonads presents different infectious organisms within the cell such as, virus like particles and bacteria. Among them, mycoplasma-like organisms are the most easily detectable in some *Trichomonas* isolates.

The genus *Mycoplasma* presents the smallest organisms lacking cell walls that are capable of self-replication and cause various diseases in humans, animals, and plants. Although mycoplasmas are recognized primarily as extracellular parasites or pathogens of mucosal surfaces, recent evidences suggested that

certain species may invade the host cells. They have also been recognized as a major contaminant of cell cultures where they thrive in close association with host cell plasma membrane and may cause drastic alterations (Stanbridge, 1982).

The aim of the present study is to further characterize this relationship between these two human pathogens that even being long time known, but still not well defined. Since it is known that *Trichomonas* is used to feed from bacteria, it is not clear whether *Mycoplasma* or *Trichomonas* benefits from one another or if they work together in a co-parasitism.

By means of electron microscopy, thin sections observations of these organisms revealed that they present a pleomorphism characterized by a size ranging from 90 to 200nm, probably due to its lack of a rigid cell wall, which may favour their adhesion and fusion with the host membrane (Scholtyseck *et al.*, 1985). This variation in shape was distributed in ovoid, spherical and peak-like protrusion forms. Once in a while some budding sites could be seen and also, many places of adherence invaginations visible on the *Trichomonas* surface. On some of the budding vesicles we could also visualize an electron dense area, which could be nucleic acid material, strongly suggesting that the mycoplasma-like organism may have some kind of cycle within the *Trichomonas* cell. Mycoplasmas also showed to cause some damage to the host, like intense cytoplasmic vacuolization sometimes harboring several mycoplasma-like structures.

Its has been shown for many years that the co-existence of different sexually transmitted diseases in the same individual it is quite common, vaginal infections by *T. vaginalis* were associated with *Mycoplasma fermentans* since 1985 by Scholtyseck *et al.*

It is still unknown if these new observations have significance with respect to virulence or pathogenicity of *T. vaginalis*, however we cannot discard the possibility of trichomonads playing the role of a new vector for infectious agents. We are directing our efforts towards the discovery of new labeling techniques and markers that could help us to elucidate these questions. Further studies with immunoelectron microscopy will be needed to confirm this possibility and to extent our knowledge on this relationship.

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### BC3 - ENDOCYTIC PATHWAY IN PHAGOCYtic AND NON-PHAGOCYtic CELLS DURING THEIR INTERACTION WITH *TRYPANOSOMA CRUZI*

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The internalization of small molecules, macromolecules, large particles and even whole cells is a process collectively called endocytosis. Following internalization, endocytic vesicles fuse with sorting endosomes localized throughout the cytoplasm, where several members of the Rab family of Ras related small GTP-binding proteins participate in the transport events such as budding, docking and vesicle fusion. The intracellular protozoan pathogen *Trypanosoma cruzi* causes Chagas' disease in humans, a chronic inflammatory conditions characterized by cardiomyopathy and digestive disorders. As (i) several alterations have been reported during the infection of host cell by parasites and (ii) endocytosis has important roles in cellular events, the aim of the present study is characterize the endocytic pathway in professional phagocytic (peritoneal macrophages-PM) and non-phagocytic cells (cardiomyocytes-CM) during their in vitro interaction with *T. cruzi*. We started our studies by analyzing the fluid-phase endocytosis in uninfected host cells through fluorescent assays using dextran conjugates (FITC-Dx) by ultrastructural approaches using albumin adsorbed to gold particles (BSA-Au). After 5-15 min of incubation at 37°C, the fluid-phase ligands were noted within early endosomes mostly localized in the

periphery of both CM and PM. Longer periods of incubation resulted in the perinuclear accumulation of the ligands in late compartments in both cell types. We next investigated the distribution of early and late endosomes by fluorescent assays using monoclonal antibodies raised against Rab 7 (marker for late endosomes-LE), Rab 11 (recycling endosomes-RE) and EEA 1 (sorting endosomes-SE) molecules. In uninfected CM, the EEA 1 labeling showed a punctual distribution throughout the cytoplasm and around the nuclei. The Rab 11 analysis in CM showed positive labeling in large vesicles and in tubular elements throughout the cytoplasm besides a bright perinuclear spot. When anti-Rab 7 was probed, CM displayed a bulk labeling surrounding the cell's nuclei and a spotted labeling in cytoplasm. However, during the interaction (24-48h) of non-phagocytic cells with trypomastigotes of *T. cruzi* (Y and Dm28c stocks) we observed a striking decrease and almost loss of the LE and RE expression in the host cells. As expected, the infection with both parasite stocks also altered the usual distribution and number of FITC-Dx-labeled late compartments in infected-CM. Flow cytometry and ultrastructural approaches are underway in order to deep analyze and compare the altered expression of endocytic compartments in both phagocytic and non-phagocytic cells during their invasion by *T. cruzi*. The observed endocytosis impairment during the infection of host cells by the parasite can contribute to the overall physiologic failure by modifying normal incoming of nutrients as well as interfering with other important events related to the endocytic pathway.

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#### **BC4 - REDISTRIBUTION OF CYTOPLASMIC ORGANELLES FROM PHAGOCYtic AND NON-PHAGOCYtic CELLS AFTER INVASION BY *TOXOPLASMA GONDII*: A STUDY BY LASER SCANNING MICROSCOPY.**

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*Toxoplasma gondii*, the causative agent of toxoplasmosis, is an obligatory intracellular parasite that establishes a most unique relationship with its host and is capable of active penetration and multiplication in any nucleated cell of warm blooded animals. Its survival strategies include the partial exclusion of host cell membrane proteins, what allows the escape from fusion of the parasitophorous vacuole with host cell lysosomes, and the rearrangement of host cell organelles in relation to the parasitophorous vacuole. In this work we report the rearrangement of host cell organelles (mitochondria and endoplasmic reticulum) around the parasitophorous vacuole of two distinct cell types: a phagocytic and a non-phagocytic cell. We used the mitochondrion-selective dye CMXRos (Mitotracker) and the carbocyanine dye DiOC6 in high concentrations (2mg/ml) to label mitochondrion and endoplasmic reticulum of LLCMK2 cells (epithelial cells from *Macaca mulata* kidney), and CF1 mice peritoneal resident macrophages, both challenged with *Toxoplasma* and allowed to interact for 1 and 24 hours. Under Laser Scanning Microscopy observation Mitotracker labeling showed filamentous mitochondria all over the cytoplasm. In some cells, specially 24 hours after challenge, enhanced fluorescence around the PV was observed, but could be the result of PV growth pushing mitochondria around it. This was observed both in LLCMK2 cells and in macrophages. Ultrathin sections of infected cells observed at the transmission electron microscope also did not show strict association of mitochondria and PV membrane. So, our results on organelle association to the PVM agreed less with other authors when mitochondria were considered both for macrophages and LLCMK2 cells. On the other hand, in cells labeled with DiOC6 an area of higher fluorescence around the PVs was always observed, showing that association of ER profiles with the growing PV, as observed by electron microscopy, indeed occurs in all cell types. These results indicate that the organelle association with the PVM, described

by Sinai et al. (*J Cell Sci.* 17, 2117[1997]) may not be mandatory for all cell types, at least in what concerns mitochondria.

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#### **BC5 - THE CYTOSKELETON PARTICIPATES ON *TRYPANOSOMA CRUZI* EPIMASTIGOTES ENDOCYtic PATHWAY**

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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 *Trypanosoma cruzi*. *T. cruzi* is a very useful cell model to investigate the influence of the cytoskeleton on endocytosis, as it possesses a very special cytoskeleton. Among the most conspicuous cytoskeleton components are the subpellicular microtubules (SPMT), underneath the plasma membrane. This parasite is a polarized cell where endocytic organelles have a very well defined cytoplasmic localization, distributed from the cell anterior region, where the cytostome, the main site of endocytic cargo entry, is placed to the posterior end where reservosomes, the lysosome-like organelles, are found. As the endocytic pathway must span for such a distance along the parasite body, it is highly feasible that microtubules and microfilaments are involved in guiding endocytic transport vesicles.

We processed for transmission electron microscopy *T. cruzi* epimastigotes that have previously uptaken colloidal gold-conjugated transferrin (Tf-Au), using a special protocol to emphasize cytoskeleton structures. We could observe connections between endocytic organelles and SPMTs. We have also used 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. As stabilizing agents, we used taxol for microtubules and jasplakinolide for actin filaments. After drug treatment, cells were incubated with transferrin-fluorescein (Tf-FITC) or Tf-Au, processed and observed. We verified some ultrastructural alterations, mainly at the cytostome. Unexpectedly, the connections between endocytic organelles and SPMTs were maintained in oryzalin and taxol treated cells. We have also quantified endocytosis using radioiodinated transferrin (<sup>125</sup>I-Tf) in the presence of the drugs. Oryzalin and taxol caused a decrease of about 50% in ligand 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 Tf-Au, cytochalasin treatment completely blocked ligand entry, entrapping Tf-FITC and Tf-Au at the cytostome and rendering <sup>125</sup>I-Tf accessible to trypsin digestion. We demonstrated, for the first time, that epimastigotes do recycle internalized Tf. The drugs, mainly the stabilizing ones, affected recycling of <sup>125</sup>I-Tf to the plasma membrane. Degradation of transferrin inside reservosomes also occurred and was almost completely blocked by cytochalasin.

In conclusion, endocytic organelles are connected to SPMTs that may guide their positioning. Actin filaments seem to be implicated in the initial events of endocytosis in epimastigotes. Transferrin recycling may depend on both cytoskeleton components – microfilaments and microtubules.

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**BC6 - IMMUNOCYTOCHEMICAL LOCALIZATION OF THE C-TERMINAL EXTENSION FROM THE CYSTEINE PROTEINASE LPCYS2 OF LEISHMANIA (L.) PIFANOI.**

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Little is known about protein sorting in trypanosomatids. We are studying the mechanisms of lysosome sorting of cysteine proteinases in leishmania. Cysteine or thiol proteinases have been identified in a variety of pathogenic microorganisms and have been implicated in processes that can be important for infection and virulence. Cysteine proteinases are highly conserved among trypanosomatids. These proteinases have three characteristic domains: the prepro sequence, the pre part being a putative signal peptide, the pro domain containing a short peptide responsible for targeting to the megasoma; the second one is the catalytic moiety, and the last one is a C-terminal extension, the function of which, until the moment, is not known. In previous studies, immunolocalization using a monoclonal antibody anti C-terminal extension, had shown the presence of this domain in the lysosome. We have produced a polyclonal antibody against this domain and used it to determine the final localization of the cysteine proteinase C-terminal extension in *Leishmania*. *Leishmania* axenic amastigotes, when exposed to this antibody, showed lysosomal labeling and also strong labeling of the flagellar pocket. Interestingly, when *Leishmania (L.) pifanoi* in early stages of macrophage infection were labeled, the C-terminal extension localized mostly to the surface of the parasite. On the other hand, a control using an antibody against the catalytic domain of Lpcys2, marked the lysosome and, to a lesser extent the flagellar pocket, as expected. This indicates that the cysteine proteinase suffers a gradual processing in the lysosome, preserving its active mature region inside this organelle, and secreting the C-Terminal domain through the flagellar pocket route. Nevertheless, there seems to be an accumulation of this domain in the flagellar pocket in the culture forms of the parasites, and a release of the C-terminal domain after contact with macrophages. This route of secretion may be used by the parasite to discard proteins that possibly already fulfilled their metabolic function and are no more necessary, or, on the other hand, it may also be that some of these proteins or proteolytic fragments have an role at the immunoregulation level, helping parasites to survive in the hostile environment of the mammalian host.

**BC7 - ENDOCYTIC PATHWAY IN EVOLUTIVE FORMS OF T. CRUZI: CHARACTERIZATION OF THE COMPARTMENTS**

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Endocytosis represents the uptake of extracellular material through multiple roads, including the phase fluid, receptor-mediated endocytosis and the phagocytosis. Endocytic pathway of evolutive forms of the *T. cruzi* is not well known, controversies exist about the existence of early endosomes in these parasites. In the present study, we investigate evolutive forms of *T. cruzi* (Y strain), the expression and the distribution of early (sorting and recycling) and late endosomes through the expression of specific GTPases by electron microscopy and flow cytometry approaches. Bloodstream trypomastigotes were obtained from *T. cruzi* infected Swiss mice [1], epimastigotes from axenic medium

and amastigotes from the supernatant of J774G-8 macrophage cell lines [2]. For ultrastructural characterization, the parasites were washed and fixed with 0.01% paraformaldehyde (PFA), 0.2% glutaraldehyde, 0.1% picric acid, dehydrated in methanol crescent series and then embedded in Lowicryl resin. Unstained ultrathin sections were incubated for 1h/37°C in a blocking buffer and further incubated for another 1h/37°C with monoclonal antibodies against Rab7 (for detecting late endosomes), Rab 11 (for recycling endosomes) and EEA1 (for sorting endosomes). After washing the samples were incubated for 30 min with the secondary antibody (goat anti-rabbit IgG) coupled to 5nm colloidal gold particles and then analyzed by transmission electron microscopy using EM10C Zeiss microscope. For flow cytometry assay, the parasites were fixed in PFA 0.2% and incubated with specific antibodies, followed by incubation with the secondary antibody (anti-rabbit IgG-TRITC) and analyzed in a FACSCalibur. All the negative controls were performed by the omission of the primary antibody. Ultrastructural analysis of the anti-EEA1 showed a dispersed labeling in the whole cytoplasm mostly near the cell periphery and within the flagellar pocket in both amastigotes and trypomastigotes. The staining with anti-Rab11 in amastigotes and trypomastigotes forms were localized close to the nucleus, kinetoplast. The labeling with anti-Rab 7 were preferentially visualized near the nucleus and the kinetoplast of the parasites. Our data showed that, the percentages of positive parasite population for each labeled endosome were similar among epimastigotes and amastigotes whereas the trypomastigotes forms displayed lower labeling, suggesting and confirming literature data concerning the higher endocytic activity in the former parasites [3]. Our data also suggest the expression of early endosomes corroborating the data of Porto-Carreiro et al (2000). Another approaches are under way to deep investigate and compare the endocytic pathway in the three evolutive stages of *T. cruzi*.

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**BC8 - DIVIDING LEISHMANIA (L.) AMAZONENSIS AND HERPETOMONAS MEGASELIAE PRESENT A FLAGELLA CONNECTOR**

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The cytoskeleton of trypanosomatids presents some particularities, such as the subpellicular microtubules (SPMT), a cage-like parallel array of singlet microtubules, which are connected to each other and to the plasma membrane by short proteic filaments. The SPMT is a stable network that confers cell shape and mechanical resistance. The trypanosomatids owns a single flagellum that emerges from the flagellar pocket. In addition to the classical "9+2" microtubule axoneme, the flagellum contains a lattice-like structure named paraflagellar rod (PFR) which runs alongside the axoneme. The PFR is evolutionary conserved among the members of the Kinetoplastida (except for the endosymbiont-bearing ones) and immunologically relevant. Recently it was described, exclusively in the procyclic form of *Trypanosoma brucei*, a new structure during cell division that attaches the new flagellum to the old one, the flagella connector (FC). The FC was described with three subcomponents: a "fuzzy" component at the distal tip of the new flagellum, a short link structure and a plate-like lamellar domain. The FC connects the new flagellum to the lateral side of the old flagellum

axoneme, and not to the PFR. It remains present even in *T. brucei* lacking flagellum-cell body attachment, and it is resistant to detergent extraction and high  $Ca^{2+}$  concentration treatment.

In this work we report the presence of a flagella connector in promastigote forms of *Herpetomonas megaseliae* and *Leishmania (L.) amazonensis*. We verified the presence of FC in 97% and 75% of *H. megaseliae* and *L. (L.) amazonensis* dividing cells, respectively. Using high-resolution field emission scanning electron microscopy (FESEM) we observed that the FC anchors the tip of the new flagellum since it shows up from the flagellar pocket and runs forward along the old flagellum until close to its distal end. In negative staining, it was possible to readily observe that the connector has a single detergent-resistant amorphous component apposed to the old flagellum axoneme. The new flagellum appears to be thinner than the old one and seems to have the PFR not completely assembled. However, immunocytochemistry using antibodies anti-PFR A (L8C4) and anti-PFR A and PFR C (L13D6) revealed both PFR major proteins distributed in the PFR of the new flagellum.

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### BC9 - ISOLATION OF LIPID DOMAINS FROM THE MEMBRANES OF *TRYPANOSOMA CRUZI* RESERVOSOMES

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Reservosomes are acidic compartments that store nutrients, found in epimastigote forms of *Trypanosoma cruzi*. They were first described as multivesicular bodies (De Souza et al, 1978 *Exp. Parasitol.*, 45:101). Further studies (Soares and De Souza, 1988 *J Submicrosc Cytol Pathol*, 20:349) using ultrastructural cytochemistry and stereology characterized reservosomes as single-membrane bound organelles ranging from 0.4 to 0.6  $\mu$ m in diameter that lack true internal vesicles and contain lipid inclusions dispersed in an electron-dense protein matrix. The lipid inclusions may present unusual aspect, forming sharp angles that sometimes seem to rupture the organelle. The frontier between lipids and the dense protein matrix may assume the trilaminar aspect of a lipid bilayer. It is relevant to investigate if reservosomes present or not internal membranes when we discuss the participation of Golgi elements and early endosomal vesicles in the formation or renewal of the organelle. To start answering this question, intact epimastigotes were slam frozen and freeze-fractured. Reservosomes fractured inside intact cells present flattened lipid inclusions, similar to those usually found in reservosome ultrathin sections, as well as round inclusions, resembling internal vesicles. We have recently isolated reservosomes (Cunha-e-Silva *et al.* *FEMS Microbiol. Lett* 7-14, 2002) and, in an initial lipid analysis, found high levels of colesteryl ester and ergosterol, as well as phosphatidylcoline and phosphatidylethanolamine as the major phospholipids. The reservosome purified fraction was submitted to 0.25% Triton X-100 for 30 minutes at 4°C or 37°C and layered on the top of a continuous sucrose gradient. Transmission electron microscopy of the fractions obtained showed that, while detergent treatment at 37°C completely solubilized reservosome membranes, at low temperature detergent treatment rendered a membrane preparation formed by planar membranes, akin to the trilaminar profiles found in the interior of intact organelles. Alternatively, reservosome membranes were prepared by ultrasonic disruption of purified reservosomes previously incubated in 150 mM sodium carbonate pH 11 at 4°C for 1h. Using this protocol we obtained a preparation where planar membrane profiles and round closed membrane vesicles coexist. Lipid analyses of reservosome membrane fractions are in course. Based on ultrastructural data and differential detergent solubility, we suggest that reservosomes possess internal membranes

with a distinct composition that may reflect function and origin different from the organelle external membrane.

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### BC10 - HEME REQUIREMENT AND ITS POSSIBLE INTRACELLULAR TRAFFIC IN *TRYPANOSOMA CRUZI* EPIMASTIGOTES

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*Trypanosoma cruzi*, the etiologic agent of Chagas disease, is transmitted through triatomine insects vectors during the blood-meal on vertebrate host. These hematophagous insects usually ingest in a single meal about 10 mM heme bound to hemoglobin. Heme is a powerful generator of reactive oxygen species, including free radicals, and can damage a variety of biomolecules. *T. cruzi*, in the course of their evolution history, had to develop adaptations to avoid the deleterious effects of high concentrations of free heme found in this environment and there is a large lack in literature about its mechanisms of uptake, its effects and degradation inside the parasites. The first transformation into epimastigotes occurs in the stomach and initiates few hours after parasite ingestion. We have been investigating the effects of hemin on *T. cruzi* growth. Hemin concentration in the medium varied from 0 to 1 mM. Addition of hemin drastically increased the parasite proliferation in a dose-dependent manner. Ultrastructural analysis of parasites grown in high heme concentration are in course. Pd - Mesoporphyrin IX (an analogous of heme: Fe - Protoporphirin IX) intrinsic fluorescence was used as a label to trace the fate of heme taken up by the parasite. We followed the time course of Pd-mesoporphyrin IX internalization in parasites from three to five-day-old cultures incubated with globin-Pd-Mesoporphirin IX. The fluorescence signal was initially associated with anterior vesicle compartments, reaching reservosomes after less than a minute of incubation. On the other hand, when eight-day-old parasites were starved by a 24 hour incubation in medium without serum, the images showed intense fluorescence in the kinetoplast. Taken all together, our data suggest the need of heme in the development of *T. cruzi* and points to the existence of a specific pathway for heme absorption from media, which would be consistent with the utilization of host heme for assembly of mitochondrial proteins of the parasite. Besides this we have also described the use of Pd-Mesoporphirin IX, a new tool to study cell metabolism of heme.

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### BC11 - MEMBRANE FORM VSG IS SECRETED IN VESICLES BY *TRYPANOSOMA BRUCEI*

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African trypanosomes display 10 million copies of a glycoprotein (VSG) which is anchored to the plasma membrane by glycosylphosphatidylinositol (GPI) anchor. A GPI specific phospholipase C (GPI-PLC), by clipping off the

dimyristoylglycerol from the membrane bound VSG (mfVSG), promotes VSG shedding in a soluble form (sVSG). This process can be monitored by immunodetection of an epitope called CRD on the sVSG and also by loss of mfVSG anifilicity assessed by Triton X-114 phase separation. It has been proposed that access of GPI-PLC to its substrate requires tetramerization and depends on thioacylation occurring within a cluster of three cysteine residues (Paturiaux-Hanocq et al., JBC 275:12147, 2000; Armah et al. JBC 275:19334,2000). Indeed, a trypanosome mutant whose GPI-PLC gene has been modified on the acylated cysteines (Triple Mutant, TM) lost the hallmark behaviour of releasing VSG upon hipotonic lysis. Incubation of live wild type (WT) trypanosomes for up to 60 min at 4°C and 37°C in saline phosphate buffer pH7.5 supplemented with glucose (PSG) indicated that both s and mfVSG were released in the supernant in a time and temperature dependent manner. On the other hand, GPI-PLC minus mutant (PLC<sup>-</sup>) released only mfVSG and TM released mostly intact VSG, with just a small fraction of CRD positive VSG signalling a mildly functional enzyme. WT parasites released about 0.6% of total <sup>125</sup>I-labelled VSG after 60 min at 37°C whereas TM and PLC<sup>-</sup> mutants released 10 times less VSG. Aiming to examine whether mfVSG was released in vesicles, the material shed by both WT and PLC<sup>-</sup> trypanosomes was submitted to 100.000g centrifugation for 60 min. Analyses of the pellet revealed VSG which was CRD negative unless treated with GPI-PLC and transmission electron microscopy of material fixed either prior or following ultracentrifugation revealed a quite homogeneous population of vesicles with about 60nm of diameter, obviously containing some type of material in its lumen and covered with a slightly electrondense material resembling VSG. Surprisingly an occasional “filopodium-like” structure with a “beaded” aspect was observed, suggesting that filopodia could be fragmented into vesicles. Immunolabelling experiments are being carried out to detect VSG on the vesicles released *in vitro* and also on those recovered from infected mice sera. We hypothesize that the release of mfVSG-bearing vesicles is physiologically relevant firstly, to allow the GPI anchor with its lipid moiety to modulate the host immunosystem (Magez et al. J. Immunol. 160:1949, 1998), and secondly, fostering the transference of VSG to erythrocytes (Rifkin et al., PNAS 87:801, 1990) following vesicle fusion, a possibly significant event in the context of African trypanosomiasis pathogenesis.

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## BC12 - GRISEOFULVIN INDUCES CELL DEATH BUT NOT MICROTUBULES DEPOLYMERIZATION IN *TRITRICHOMONAS FOETUS*

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*Tritrichomonas foetus* is an extracellular parasitic protist that inhabits the urogenital tract of cattle causing the bovine trichomonosis. Like other trichomonads species *T. foetus* possesses very peculiar cytoskeletal structures such as the costa and the pelta-axostyle complex, responsible for maintaining the cell body shape. The axostyle, a ribbon of longitudinally-oriented microtubules which runs from one pole of the cell to the other, has been subject of controversy concerning its stability. We have previously demonstrated that the axostyle is maintained through the entire cell cycle of trichomonads, and does not depolymerize [1]. Recently, Noel et al. showed the effects of the anti-fungal compound griseofulvin on the cytoskeleton of *Trichomonas vaginalis* [2]. These authors stated that the drug irreversibly affects tubulin assembly in trichomonads, suggesting thus that the axostyle is composed of labile microtubules. In view of these contradictory observations, we decided to observe

the effects of this anti-fungal on *T. foetus* by other methods.

We took advantage of complementary techniques in order to observe the effects of griseofulvin on *T. foetus* ultrastructure. The reversibility of the compound was also tested. After incubation with 50µg/ml griseofulvin at different times the cells were fixed and processed for fluorescence microscopy, scanning (SEM) and transmission (TEM) electron microscopy. By SEM and TEM we found that the drug induced a cell death with characteristics commonly observed during apoptosis, such as membrane blebbing. Autophagic features were also observed (eg. intense cytoplasmic vacuolization). The axostyle-pelta complex was fragmented, but not depolymerized. By light and fluorescence microscopy membrane blebbing and axostyle fragmentation were found, as well as nucleus condensation and fragmentation. Phosphatidylserine was translocated from the inner to the outer leaflet of the plasma membrane, as detected by annexinV. The effects of the drug were time-dependent, and reversible until six hours of incubation.

Altogether the data herein presented show that griseofulvin induces a type of cell death in *T. foetus* with characteristics resembling both apoptotic and autophagic processes. Besides, the effects of the anti-fungal griseofulvin can be reversible and it never leads to depolymerization of microtubular structures, corroborating our previous observations that the axostyle-pelta complex is composed of stable microtubules [1].

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## BC13 - EXPRESSION OF ANIONIC SITES IN THE WALL OF TISSUE CYSTS OF *TOXOPLASMA GONDII*

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*Toxoplasma gondii* is an obligate intracellular protozoan parasite, member of phylum Apicomplexa that have ability to infect a wide range of hosts, including mammals and birds. (Coppin et al, 2003). To survive within infected hosts, *T. gondii* undergoes profound metabolic and morphological changes by differentiation into a cyst characterized by its resistance to the immune system and chemotherapy (Bohne et al, 1999; Lyons et al, 2002). The cyst wall is thought to be important in providing stability to the tissue cysts and in facilitating cyst persistence in chronically infected hosts. However the composition and biogenesis of the cyst wall in *T. gondii* has not been fully elucidated (Coppin et al, 2003).

Our present aims were to study the expression of anionic sites in the wall of tissue cysts. C57BL/6 mice were infected with 50 cysts/animal of *T. gondii* ME-49 strain type II. After 4-8 weeks of infection, the brains were harvested, homogenized and purified in dextran gradients. The cysts were incubated with two markers cationic: cationized colloidal gold (C-Au) and cationized ferritin (CF) for 20 min 4°C and additionally for 1, 6 and 24 hours at 37°C followed by processing for transmission electron microscopy. Assays were also performed

using fixed cysts. The incubation of tissue cysts at 4°C with CF showed a homogeneous distribution of anionic sites on the wall cyst, which was followed by their incorporation after raising the temperature (37°C). The CF internalized was localized within vesicles, tubules and in the cyst matrix and sometimes, in close proximity or in direct contact with bradyzoite membrane inside the tissue cysts. Fixed cysts presented lower labeling when compared with non-fixed cysts. The expression of anionic sites using cationized colloidal gold particles showed a discontinuous labeling at 4°C, but after 6 and 24h at 37°C the particles were localized attached on the cystic wall.

The endocytic activity of the cystic wall revealed by anionic sites incorporation using cationized ferritin argues for its role during uptake of the nutrients by tissue cysts and represents a potential target for chemotherapy tests.

Supported by CNPq, FAPERJ, UERJ, and IOC/FIOCRUZ.

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### BC14 - ANALYSIS OF THE SHEDDING PROCESS IN TRIPOMASTIGOTE FORMS OF *TRYPANOSOMA CRUZI*

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Cleavage or shedding of functional proteins is an important process on cell surface modulation and particularly important in parasite infections, where different mechanisms for evading the immune response are requested. Previous studies have shown that two kinds of host immunoglobulins can interact with the surface of *Trypanosoma cruzi*, a non-specific bind to the parasite surface through either Fc or Fab fragments of IgG, and a specific human and animal IgG antibodies, capable to induce mobility of surface antigens of live blood forms to form a cap in the anterior and posterior poles of the parasite in a process dependent of parasite strain, time, temperature and inhibited by sodium azide. Aggregated membrane components shedded from parasite surface, explain the immunopathological alterations described in the kidney of mice infected. However this process was described as a spontaneous process involving a plasma membrane vesiculation, results obtained by previous researches do not exclude that some antigens may have a differential rate of shedding or be enhanced in the presence of some substances or molecules. The aim of present study is the analysis of the shedding process in two different strains of tripomastigote forms of *T. cruzi*, and under the influence of different molecules. Briefly, tripomastigote forms from CL Brener clone and Y strain, were incubated in the presence of non-related IgG-gold complexes during 30 minutes at 37°C or 4°C. Experiments were also realized in the presence of Cationized Ferritin (FeCat) or with Concanavalin A (ConA) with previous incubations for 30 minutes at 4°C and subsequently during 10, 20 and 30 minutes at 37°C. Some samples were pre-fixed or pre-treated with sodium azide. All cells were processed and observed in scanning and transmission electron microscopy. In the presence of non-related IgG, parasites of both strains showed a small pattern of shedding when incubated at 4°C. Although, at 37°C, an intense shedding process could be observed with numerous vesicles distributed on the cell body of both strains. Vesicles aggregated could also be observed in extracellular milieu in both experimental conditions. Experiments realized with FeCat showed a discrete shedding process in samples incubated at 4°C. Despite that, when these samples were submitted to a second incubation of 30 minutes at 37°C, we could observe a little increase of the process.

Similar results were observed in both strains. In the presence of Con-A, CL Brener tripomastigote forms didn't show shedding vesicles as described for FeCat. Complementary experiments using Con-A or PMA still in process.

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### BC15 - PRODUCTION OF AN ANTIBODY AGAINST *L. (L.) AMAZONENSIS* ARGINASE

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*Leishmania* is an intracellular protozoa that, to survive inside the host macrophage, escapes from its microbicidal mechanisms. Amongst these mechanisms are the production of nitric oxide (NO) and superoxide radicals (Bogdan et al., 1996). The production of NO by inducible nitric oxide synthase (iNOS) requires L-arginine as substrate. In addition to, arginase requires this aminoacid to generate ornithine and urea. Arginase may play an important role in parasite survival by decreasing the concentration of the iNOS's substrate and reducing the NO generation, as well as in the production of ornithine, a precursor for polyamines synthesis and consequently involved in DNA synthesis and cell proliferation (See Alves et al, this meeting). Moreover, there is a study showing that the inhibition of arginase by N<sup>0</sup>-hydroxy-L-arginine enhances NO production and increases the killing of the parasite (Iniesta et al., 2001).

In order to better understand the physiological role of arginase and its importance in *Leishmania*'s infection, the subcellular localization of the enzyme should be known. In the present communication, we describe the first step to get this localization, the production and characterization of a specific antibody against arginase.

The arginase coding gene from *L. (L.) amazonensis* was described and the open reading frame was cloned into a modified expression vector, pRSET, and used to transform *E. coli* BL21. Arginase was purified for an IPTG-induced culture of transformed bacteria. BALB/c mice were then inoculated with 18,5µg of purified arginase and complete adjuvant subcutaneously. After 30 days, the burst was performed by another inoculation using 18,5µg of purified enzyme with incomplete adjuvant. The antiserum was prepared after collecting the animals blood. To test its specificity for arginase, the antiserum obtained were used in Western blot experiments, containing PAGE fractionated cellular extracts of *L. (L.) amazonensis* as well as the transformed *E. coli* producing arginase, with and without IPTG induction. A significant stained band, with the same apparent molecular weight of the arginase was obtained. This result indicates that the antiserum obtained recognizes arginase with enough specificity.

Cytological preparation will now be submitted to the antiserum to localize the enzyme in different conditions of infection.

Supported by FAPESP and CNPq.

### BC16 - *TOXOPLASMA GONDII*: ENDOCYTIC ACTIVITY

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*Toxoplasma gondii*, an obligate intracellular parasite, has elaborated a battery of specialized secretory organelles (micronemes, rhoptries and dense granules) that mediate host cell attachment and invasion, and the formation and maturation of parasitophorous vacuole [1]. Endocytosis mechanisms are poorly known in

these Apicomplexan parasites [2]. Little information about the lysosomal system in *Toxoplasma* has been reported, which raises a number of questions regarding parasite's basic cellular processes. The only acidic compartment described in *Toxoplasma* tachyzoites is the forming and the mature rhoptries [3]. Some extra cellular tachyzoites have been observed to slowly internalize the fluid phase endocytic tracer, horseradish peroxidase (HRP), through vesicles formation at the base of the micropore, the single structure that has been identified in nutrient acquisition in *T. gondii* [4]. The present work was undertaken to study the endocytic activity of *T. gondii*.

RH strain *Toxoplasma* tachyzoites were obtained from peritoneal exudates of Swiss mice 3 days after parasites inoculation. Exudates were washed with PBS (Phosphate Buffered Saline) and incubated for 20 min at 37°C with 100 mg/ml HRP-Au at 37°C and after 5 min to 24 h, the samples were washed twice with PBS and parasites were fixed for 30 min, at 4°C in a solution of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, supplemented with CaCl<sub>2</sub>. The parasites were washed three times in the same buffer and then post-fixed in 1% OsO<sub>4</sub>. The cells were dehydrated in crescent concentrations of acetone and embedded in Epoxy resin. Thin sections were obtained, stained with uranyl acetate and lead citrate and examined by transmission electron microscopy, in a Zeiss EM10.

The ultrastructural analysis showed with relative frequency, HRP-Au inside rhoptries. It was commonly observe in the same parasite more than one rhoptry containing Au-particles. We obtained up to now no evidences of HRP-Au particles uptake through the micropore or any other parasite organelle. Our preliminary data suggest that rhoptries are involved in nutrient acquisition by *Toxoplasma*, despite their well-known role as secretory organelle during host cell invasion. Further studies will be developed to elucidate the mechanisms involved in the nutrients uptake by *Toxoplasma*, a phenomena not yet understood and of great relevance for the development of drug delivery targeting into the tachyzoites.

Supported by CNPq, FAPERJ and IOC/FIOCRUZ.

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#### BC17 - INVASION AND EGRESSION FROM BLOOD MONOCYTE OF AN *HAEMOGREGARINE* SP. FROM THE LIZARD *AMEIVA AMEIVA* (REPTILIAN: TEIIDAE).

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Parasites from the apicomplexan group have an obligate intracellular existence. Thus, active invasion and egression of host cells are a necessity and have been described for a few species. However, for a better understanding of this group, new descriptions of these processes from different species are necessary. Gliding is a process used by these parasites to move through the environment and to invade cells. We have been working with an Amazonian haemogregarine found in blood monocytes of the *Ameiva ameiva* that induces monocytosis positively correlated with parasitemia. Here we describe the final moments of the evasion and the whole invasion process of this parasite. Blood was collected by cardiac puncture and leukocytes separated by centrifugation. Live cells were mounted under a slide, observed and photographed under a

Zeiss Axiophote using an immersion 100 x objective. Parasite was seen attached by its posterior end with most of its body out of the cell rotating furiously. It was impossible to determine the rotation side because of the high speed. After a few seconds, the parasite started to pull a portion of the monocyte plasma membrane, which formed a "cup" around the posterior portion of the parasite body. The parasite also moved down stretching the plasma membrane cup that increased in size. After 2 minutes, the parasite was released from the plasma membrane of the monocyte and immediately stopped the rotation movement and started the gliding motion through the slide. The other observation was the infection process. Noninfected monocytes were spread at this time. The parasite approached the monocyte using the gliding motion. Its anterior portion moved from one side to the other in a touching process. There was no evidence of attachment to a special region of the monocyte membrane. Suddenly the conoid extruded and the parasite invaded the monocyte within two minutes. Later the spread monocyte started to retract. After twenty-five minutes, all the monocyte filopodium retracted and the cell rounded up. Fifty-seven minutes later, the infected monocyte assumed a round shaped. These results show that the egression process used by this parasite is unique and is based on rotating movement. It also supports the notion that the gliding motility capacity is conserved among apicomplexan parasites and illustrates how this invasion process alters the monocyte spreaded morphology.

Supported by: CAPES, CNPq, FAPERJ, PRONEX.

#### BC18 - MODULATION OF NEUTROPHIL ADHESION TO VASCULAR ENDOTHELIAL CELLS BY INACTIVE MEMBERS OF *TRYPANOSOMA CRUZI* TRANS-SIALIDASE FAMILY

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*Trypanosoma cruzi*, the etiologic agent of Chagas' disease, is a major cause of acute and chronic myocarditis and cardiomyopathy in endemic areas in Latin America. Host resistance to *T. cruzi* infection depends on both innate and acquired immunity. Polymorphonuclear neutrophils (PMN) constitute the first line of defense against invading microorganisms, however, they have been considered one of the major contributors to host damage in subacute and chronic inflammatory states. Migration of PMN from blood is a multi-step event dependent on lectin/sialylglycoproteins interactions mediating neutrophil-endothelial cell adhesion. *T. cruzi* express on its cell surface an inactive member of *trans*-sialidase family that physically interacts with sialic acid containing molecules on host cells. Inactive *trans*-sialidase binds to molecules containing sialic acid on endothelial cell surface inducing NF-κB activation and adhesion molecules expression. In this work we aim to elucidate the roles of iTS in PMN recruitment during Chagas' disease. Using immunofluorescence microscopy we demonstrate that iTS binds α<sub>2</sub>,3-sialic acid on CD43 from human PMN surface inducing actin cytoskeleton mobilization and reorganization. iTS binding to CD43 inhibits PMN adhesion to activated endothelial cells, *in vitro* suggesting that members of the TS family are involved in the recruitment of circulating leukocytes and play a major role in the inflammatory response during *T. cruzi* infection, contributing to the pathogenesis of chagasic disease.

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### BC19 - PARASITIC INFECTION DISRUPTS CX43, BUT NOT TIGHT JUNCTION PROTEINS IN AN EPITHELIAL CELL LINE

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We have previously shown Connexin 43 (Cx43) gap junction protein to be associated with the tight junction protein ZO1 in membranes of cardiac cells, and that infection with the protozoan parasite, *Trypanosoma cruzi*, the causative agent of Chagas disease, disrupts these proteins in the membranes of these cells. Interestingly, when infected cultures of MDCK type II cells (canine kidney derived cell line) were examined, although dye spread from the infected cell to its neighbors was completely abolished (indicating disruption of gap junctional communication), neither surface membrane ZO1 labeling nor transepithelial resistance of the monolayer were affected (indicating that the infection does not disrupt tight junctions). We therefore decided to examine the effect of *T. cruzi* infection on the expression of Cx43 and 4 members of the family of Tight Junction proteins: Zonula Occludens 1 (ZO1), Zonula Occludens 2 (ZO2), Claudin and Occludin in cultures of epithelial MDCK type II cell line. Cultured cells were infected with the Y strain of *T. cruzi* at a multiplicity of infection of 20 parasites/cell and the degree of coupling and surface expression of Cx43, ZO1, ZO2, Claudin and Occludin were examined by immunofluorescence under conventional and confocal microscopy 48-72 hours after infection. As expected infected cells in the culture were uncoupled from their neighbors, based on spread of Lucifer yellow. Surface labeling for Cx43 was markedly decreased in infected cells, but the tight junction proteins ZO1, ZO2, Claudin and Occludin were not affected. The non-infected cells in the infected cultures displayed normal expression of Cx43 with co-localization of the Cx labeling in membrane appositional areas with ZO1. We conclude that infection with *T. cruzi* disrupts Cx43 protein, but does not alter the expression of tight junction proteins in epithelial cells.

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### BC20 - FUSION OF *TRYPANOSOMA CRUZI* VACUOLES TO *COXIELLA BURNETII* VACUOLES: LIFE-CELL IMAGING STUDIES IN DOUBLY INFECTED VERO CELLS

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Background: *Coxiella burnetii*, the agent of Q fever, is an obligate intracellular bacterium that multiplies within vacuoles of phagolysosomal origin. *Trypanosoma cruzi* trypomastigotes invade a wide variety of cells and in the process recruitment of lysosomes and parasitophorous vacuole formation is observed. We have studied the fate of different *T. cruzi* trypomastigote forms after invading Vero cells persistently colonized with *C. burnetii*. When the invasion step was examined, we found that persistent *C. burnetii* infection *per se* reduced only tissue-culture trypomastigote (TCT) invasion, whereas raising vacuolar pH with Bafilomycin A1 and related drugs, increased invasion of both metacyclic and TCT trypomastigotes when compared to control Vero cells. Kinetic studies of trypomastigote transfer indicated that metacyclic trypomastigotes parasitophorous vacuole (PV) are more rapidly and efficiently fused to *C. burnetii* vacuoles. Endosomal-lysosomal sequential labeling with EEA1, LAMP-1, and Rab7 of the PVs formed during the entry of each infective form revealed that the phagosome maturation processes are also distinct.

Metodology: We have transfected Vero cells (colonized or not with *C. burnetii*) with fluorescent EGFP-tagged-LAMP-1 plasmids and observed

lysosomal dynamics under confocal microscopy. We also observed the invasion and fusion events of metacyclic trypomastigote vacuoles within transfected Vero cells colonized with *C. burnetii*. Video-microscopy was employed to observe the dynamics of parasitophorous vacuole fusion.

Results: We have observed that EGFP-LAMP-1 is recruited by metacyclic trypomastigotes in Vero cells colonized with *C. burnetii* and remained associated with metacyclic trypomastigotes parasitophorous vacuoles until their fusion with *C. burnetii* vacuoles. We also observed that metacyclic trypomastigotes parasitophorous vacuoles have an unexpected mobility, roving around *C. burnetii* vacuoles without losing GFP-LAMP protein.

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### BC21 - GAP JUNCTIONS DISTRIBUTION IN MICE EMBRYOS CARDIOMYOCYTES DURING *IN VITRO* INFECTION WITH *TRYPANOSOMA CRUZI*.

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Gap junctions are molecules belonging to the connexin multigene family that in hearts provide a low resistance pathway for electrical coupling between adjacent cardiomyocytes [Yeh, H.I. et al., *J. Histochem. and Cytochem.*, 51 259-266, 2003]. We developed an experimental system of primary culture of heart muscle cells (HMC) for studies of *T. cruzi*-cardiomyocyte interaction. Hearts of 18 days old mouse embryos were dissected, minced and incubated in a trypsin-collagenase solution. [Meirelles, M.N.L. et al., *European Journal of Cell Biology*, 41 198-206, 1986]. Previous work from our laboratory using ultrastructural and electrophysiological approaches showed that cardiac cells present the same basic characteristics found in mammalian cells *in vivo* such as specialized structures including sarco-tubular system, intercalated discs, myofibrilles, coupling interaction among the cells and spontaneous contractility. We noticed that in 3-day-old cultures that have been infected with *T. cruzi* the rate of spontaneous beating reached a peak value significantly greater than the 3-day-old control cultures, showing an increase in automaticity, higher spontaneous firing rate [Aprigliano, O. et al., *J Mol Cell Cardiol.*, 25 1265-1274, 1993].

In the present work, we investigate the distribution of gap junctions in heart cells infected or non infected with *T. cruzi*, from two different strains (Y and Dm28c) during development of the parasite's cell cycle in HMC. For the immunofluorescence studies, *T. cruzi* infected and non infected cardiomyocytes grown on coverslip were fixed for 5 min. at room temperature in 1% paraformaldehyde and were then incubated in a 1/400 dilution for 1h/37°C with a polyclonal antibody anti-connexin 43. After rinsing, the cells were incubated in a 1/800 dilution of TRITC-coupled anti-rabbit IgG. Cytoskeleton of the cells were stained with Falloidin FITC 1:200 for 30min. at 37°C. For ultrastructural cytochemistry, cells were fixed with 0.01% paraformaldehyde, 0.2% glutaraldehyde, 0.1% picric acid, dehydrated in methanol crescent series and embedded in Lowicryl resin. Unstained ultrathin sections were incubated for 1h/37°C in a blocking buffer and further incubated with the polyclonal anti-connexin 43 antibody. After washing, the grids were incubated for 30min. with the secondary antibody coupled to 10nm colloidal gold particle. The fluorescence assays showed in groups of 4-8 coupled cells the distribution of connexin in the cell's sarcolemma; highly *T. cruzi* infected cardiomyocytes showed that punctate gap junctional staining was much reduced in infected cells besides reduced fluorescence among the cells. Ultrastructural approaches showed that coupled cardiomyocytes displayed gold particles organized as round structures at the sarcolemma of adjoining cells. These studies may contribute for the understanding of how the parasite affects the functional coupling of mouse cardiomyocytes *in vitro*.



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## BC22 - MOLECULAR BASIS OF NON VIRULENCE OF *TRYPANOSOMA CRUZI* CLONE CL-14

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We investigated the properties of metacyclic trypomastigotes of non virulent *T. cruzi* clone CL-14, as compared to the parental isolate CL. In contrast to the highly infective CL isolate, which produces high parasitemias in mice by intraperitoneal or oral administration, metacyclic forms of clone CL-14 failed to produce patent infection. *In vitro*, the number of clone CL-14 parasites that entered epithelial HeLa cells, after 1 h incubation, was ~4-fold lower than that of the CL isolate and, at 72 h post-infection, intracellular replication was not apparent whereas HeLa cells infected with CL isolate contained large number of parasites replicating as amastigotes. The stage-specific surface glycoprotein gp82 plays a central role in mucosal invasion of CL isolate metacyclic forms, leading to systemic infection upon oral challenge (Neira et al., 2003, *Inf. Immun.* 71: 557-561) and is involved in host cell invasion *in vitro* (Ruiz et al., 1998, *Biochem. J.* 330: 505-511). Analysis by flow cytometry revealed that metacyclic forms of clone CL-14 express reduced levels of gp82 on the surface, although its overall expression, detectable by immunoblotting, is comparable to that of CL isolate, indicating that in clone CL-14 gp82 is mostly localized intracellularly. Otherwise, the surface profile of CL isolate and clone CL-14 was similar. Internally located clone CL-14 gp82, which is not accessible to Mab 3F6, had its reactivity augmented by permeabilization of parasites with saponin. HeLa cell entry of clone CL-14 metacyclic forms increased upon treatment of parasites with neuraminidase, which removes sialic acid from the surface glycoprotein gp35/50, a mucin-like molecule previously shown to be engaged in cell invasion of poorly infective *T. cruzi* isolates (Ruiz et al., 1998). Activity of cysteine proteinase cruzipain, reported to participate in host cell invasion and intracellular multiplication (Meirelles et al., 1992, *Biochem. Parasitol.* 52: 175-184), was similar in CL isolate and clone CL-14. Taken together, these data suggest that the lack of virulence of clone CL-14 is associated with the reduced expression of surface gp82 and the possible participation of gp35/50 in host cell invasion.

Work supported by Fapesp

## BC23 - INVASION OF MDCK EPITHELIAL CELLS WITH ALTERED EXPRESSION OF RHO GTPASES BY *TRYPANOSOMA CRUZI* AMASTIGOTES AND METACYCLIC TRYPOMASTIGOTES OF STRAINS FROM THE TWO MAJOR PHYLOGENETIC LINEAGES

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Rho GTPases have been shown to regulate three separate signal transduction pathways linking plasma membrane receptors to the assembly of distinct actin filament structures. Rho GTPases which comprises Rho (formation of stress fibers), Rac (lamellipodium), Cdc42 (filopodia), TC10, RhoG, and RhoE have been shown to mediate extracellular signals to produce distinct microfilament-

associated host cell responses. Here we examined the role of these small GTPases on the interaction between different *T. cruzi* infective forms of strains from the two major phylogenetic lineages with MDCK cells, transfected with different Rho GTPases constructs. We compared the infectivity of amastigotes isolated from infected cells (intracellular amastigotes) with forms generated from the axenic differentiation of trypomastigotes (extracellular amastigotes) and also with metacyclic trypomastigotes. Recent studies with different *T. cruzi* isolates have grouped the parasite in two major phylogenetic lineages: *T. cruzi* I associated with the domestic cycle and human disease designated and *T. cruzi* II linked to the sylvatic cycle of the parasite and other mammalian hosts. Besides the different genetic markers, *T. cruzi* I and II infective forms engage characteristic signaling pathways upon invasion of cultured cells that lead to distinct infectivities.

No detectable effect of GTPase expression was observed on metacyclic trypomastigote invasion and parasites of Y and CL (*T. cruzi* I) strains invaded to similar degrees all MDCK transfectants, and were more infective than either G or Tulahuen (*T. cruzi* II) forms. Intracellular amastigotes were complement sensitive and showed very low infectivity towards the different transfectants regardless of the parasite strain. Complement-resistant extracellular amastigotes, especially of the G strain, were highly infective for the constitutively active GTPase transfectants, particularly Rac1V12. The fact that invasion was inhibited in Rac1N17 dominant negative cells suggested an important role for Rac1 in this process. Moreover, we also observed a discrete accumulation of both actin and Rac1 protein at the sites of extracellular amastigotes invasion, reinforcing the notion that Rac1 may be involved with the process of extracellular amastigotes invasion in these cells.

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## BC24 - ROLE OF SMALL GTPASES IN *TRYPANOSOMA CRUZI* INVASION IN MDCK MUTANT CELL LINES.

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*Trypanosoma cruzi* is an intracellular parasite that modulates a great number of host intracellular responses like changes into the actin cytoskeleton and the recruitment of lysosome to its entry site. The small GTPases, RhoA, Rac1 and Cdc42 are examples of molecules that could be activated at this point of the invasion. The activation of these GTPases by signaling molecules modulates changes in the pattern of actin cytoskeleton that leads to the formation of structures like stress fibers, lamellipodium and filopodium, respectively. Here we investigated the role of the small GTPases RhoA, Rac1 and Cdc42 in the cytoskeletal rearrangement in the host cell during *Trypanosoma cruzi* infection. For that we observed the behavior of MDCK cell lines using the tetracycline repressible transactivator to regulate RhoAV14, RhoAN19, Rac1V12, Rac1N17, Cdc42V12, and Cdc42N17 expression.

*T. cruzi* adhesion and internalization indexes after 2 hours and the infection rate after 48 hours in the MDCK cell lines TET, Rac1V12, Rac1N17, RhoAV14, RhoAN19, Cdc42V12 and Cdc42 N17 were analyzed. Comparative analysis showed that Rac1V12 and Rac1N17 mutants presented the highest adhesion and internalization indexes, but the lower infection rate after 48 hours, when compared with TET control group.

Confocal laser scanning microscopy showed changes in the pattern of actin distribution in these clones suggesting that there is actin reorganization at the site of trypomastigote entry. Our results suggest that changes in the actin cytoskeleton are caused not just due to the *T. cruzi* presence but for the c-DNA expression of each transfected lineage and, this reorganization, somehow affects the parasite rate of infection. Taken together, these observations suggest that the

small GTPases RhoA, Cdc42 and mainly Rac1, act in the signaling mechanism modulating host cell responses involved in *Trypanosoma cruzi* invasion.

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## BC25 - INFECTION BY *TRYPANOSOMA CRUZI* METACYCLIC FORMS DEFICIENT IN THE EXPRESSION OF SURFACE MOLECULE GP82

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*Trypanosoma cruzi* metacyclic trypomastigotes have been reported to invade and replicate in the gastric mucosal epithelium upon oral infection. Here we analysed the process of infection by *T. cruzi* isolates deficient in the expression of gp82, the metacyclic stage-specific surface glycoprotein implicated in target cell entry in vitro and in promoting mucosal infection in mice upon oral challenge. Gp82-deficient metacyclic forms of isolates 569 and 588 infected mice by oral route, producing patent parasitemia, but to greatly reduced levels when compared to the gp82-expressing isolate CL. Metacyclic forms of both isolates expressed gp30, a surface glycoprotein detectable by MAb 3F6 directed to gp82. Otherwise, the gp82-deficient isolates displayed a surface profile similar to that of the CL isolate and also entered epithelial HeLa cells in a manner inhibitable by monoclonal antibody (MAb) 3F6, and dependent on the parasite signal transduction that involved activation of protein tyrosine kinase and Ca<sup>2+</sup> mobilization from thapsigargin-sensitive stores. Like gp82, gp30 triggered the host cell Ca<sup>2+</sup> response required for parasite internalization. Purified gp30 and the recombinant gp82 inhibited HeLa cell invasion of metacyclic forms of isolates 569 and 588 by ~90% and ~70%, respectively. Cell invasion assay performed in the presence of gastric mucin, mimicking the in vivo infection, showed an inhibition of 70-75% in the internalization of gp82-deficient isolates, but not of the CL isolate. The recombinant gp82 exhibited an adhesive capacity towards gastric mucin much higher than that of gp30. Taken together, all these findings suggest that gp30 mediates target cell entry of gp82-deficient metacyclic trypomastigotes but it fails to promote an efficient mucosal infection due to its poor interaction with gastric mucin, which may be a preceding step for parasite invasion of underlying epithelial cells.

SUPPORTED BY FAPESP

## BC26 - *TRYPANOSOMA CRUZI* TRANS-SIALIDASE ACTIVATES ENDOTHELIAL INDUCING VASCULAR ADHESION MOLECULE EXPRESSION AND RECRUITMENT OF NEUTROPHILS

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Chagas' disease, caused by the protozoa *Trypanosoma cruzi*, is a major cause of acute and chronic myocarditis and cardiomyopathy in endemic areas

in Latin America. Host resistance of *T. cruzi* infection depend on both innate and acquired immunity. Polymorphonuclear neutrophils (PMN) constitute the first line of defense against invading microorganisms, however, they have been considered one of the major contributors to host damage in subacute and chronic inflammatory states. Migration of PMN from blood is a multi-step event dependent on lectin/sialyloligosaccharides interactions mediating neutrophil-endothelial cell adhesion. Activation of NF- $\kappa$ B is associated with inflammation and expression of adhesion molecules, which are involved in the recruitment of leukocytes, a critical factor in the initiation of inflammation. *T. cruzi* expresses the active and inactive forms of *trans*-sialidase (TS), the active form transfer sialic acid residues  $\alpha$ 2,3-linked to b-galactopiranosose (b-Galp) on host donor molecules to mucin-like acceptor glycoproteins while TS inactive bind  $\alpha$ 2,3 sialic acid-glycoconjugate acting like a lectin. As the vascular endothelium is an early target to parasite invasion, here we tested the influence of *T. cruzi* TS in human bone marrow endothelial cells (HBMEC) and porcine aortic endothelial cells (PAEC). We demonstrate that inactive TS binds to HBMEC and the binding could be abrogated by the addition of  $\alpha$ 2,3-sialyllactose. Both active and inactive TSs trigger NF- $\kappa$ B activation and expression of the endothelial cell adhesion molecules E-selectin, ICAM-1 and V-CAM in both type cells. We also demonstrate that activation of endothelial cells by both aTS and iTS increases neutrophil-endothelial adhesion in PAEC. These findings suggest that members of the TS family play a major role in the inflammatory response during *T. cruzi* infection, contributing to the pathogenesis of chagasic cardiomyopathy.

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## BC27 - THE KNOCKOUT OF A SINGLE COPY ARGINASE GENE OF *LEISHMANIA (L.) AMAZONENSIS* PREVENTS ITS SURVIVAL AND MULTIPLICATION IN MOUSE MACROPHAGES.

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*Leishmania* is an intracellular parasite that escapes microbicidal mechanisms such as nitric oxide (NO) and superoxide radical production in order to survive inside host macrophages (Bogdan et al., 1996). The inducible NO-synthase (iNOS) of macrophages acts on the substrate L-Arginine to convert it to NO and citrulline. L-Arginine is also the substrate for another enzyme, arginase, to produce ornithine and urea (Krebs et al 1932). The inhibition of arginase by N-W-hydroxy-L-arginine down-regulated NO production, preventing killing of *Leishmania* inside cultured macrophages (Iniesta et al., 2001). In *Leishmania*, arginase was initially described as taking part of the urea cycle (Camargo et al., 1978). Our hypothesis is that *Leishmania* arginase could also have a major role in securing the survival of this protozoa inside macrophages by depleting the pool of L-arginine available to macrophage iNOS activity and, in consequence, decreasing NO generation.

A single copy arginase knockout *L. (L.) amazonensis* was constructed and characterized. *In vitro* and *in vivo* infection experiments in murine macrophages (BALB/c and C57BL6) showed that the *L. (L.) amazonensis* Darg::hyg/ARG mutant, in comparison with the wild type parasite, was much more readily destroyed inside macrophages and was not able to cause lesions when injected in the mouse footpad. However, experiments carried out in C57BL6 mice deficient in iNOS (iNOS-KO) showed that amastigote growth of mutant *Leishmania* in iNOS-deficient macrophages was impaired and footpad lesions did not develop when mutant *Leishmania* was injected in iNOS-KO mice (Alves et al., 2002).

In order to further investigate these findings, the *in vivo* infection experiments in iNOS-KO mice were repeated. *L. (L.) amazonensis* Darg::hyg/ARG or the

corresponding wild type *Leishmania* strain ( $10^6$  - promastigotes) were injected in iNOS-KO and WT C57BL6 mice. At selected times after infection (11 and 22 weeks) mice were killed and the footpads and draining lymph nodes (LN) removed. Parasites numbers in the LN were determined by limiting dilution technique. DNA was extracted from footpads and LN and submitted to PCR using *Leishmania* specific primers. The results show that no parasites were detected after 11 or 22 weeks in tissues from iNOS KO mice infected with the mutant *Leishmania*, in contrast to the very large number of parasites found in the mice injected with wild-type *Leishmania*. Furthermore, parasite DNA was not detected by PCR in the former group. These results indicate that the *L. (L.) amazonensis* Darg::hyg/ARG besides being more susceptible to intracellular destruction inside macrophages is not capable of establishing a lasting infection of these cells even in the absence of host NO production. This could be explained by a deficiency in the parasite's replication, probably due to insufficient polyamine synthesis. Indeed, the rate of DNA synthesis of the mutant *Leishmania*, as determined by  $^3\text{H}$ -thymidine incorporation, was much slower than that of the wild type strain.

Altogether, the results indicate that arginase is important for *Leishmania* to escape from the macrophage microbicidal mechanisms by reducing the availability of L-arginine as iNOS substrate as well as providing ornithine to the polyamines' synthesis pathway essential for DNA replication.

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#### BC28 - REGULATION OF EXTRACELLULAR MATRIX EXPRESSION DURING *TRYPANOSOMA CRUZI*-CARDIOMYOCYTE INTERACTION

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Fibrosis, an outstanding manifestation of Chagas disease, occurs as a result of exacerbated accumulation of extracellular matrix (ECM) leading to myocardium hypertrophy and heart failure (Andrade et al, 1989). Fibrosis process occurs in association with inflammatory infiltrates and degenerating cardiomyocytes (Rossi, 2001). Several reports have demonstrated that cytokines synthesized during inflammation can mediate fibrosis process. TGF- $\beta$ , a multifunctional cytokine which elicits a strong fibrogenic response is associated with Chagasic cardiomyopathy (Araújo-Jorge et al, 2002). However, the regulatory mechanisms of fibrosis remain to be elucidated.

To evaluate the kinetics of extracellular matrix (ECM) expression and the role of cytokines on its regulation, primary culture of heart muscle cells were infected with *T. cruzi*, Y strain, and the interaction was interrupted after 24 to 96h. The involvement of TGF- $\beta$  in the modulation of ECM was investigated by treating both uninfected and *T. cruzi*-infected cardiomyocytes with 5, 10 e 15 ng/ml of this cytokine. The fibronectin (FN) and laminin (LN) expression was detected by indirect immunofluorescence and the samples were analyzed by conventional and confocal laser scanning microscopy.

An intense deposit of FN and LN was observed in uninfected cardiomyocytes. No substantial alterations in the FN and LN expression patterns were detected after 24 and 48h of *T. cruzi* infection. However, fibronectin was absent or present only in reduced amounts in highly infected cardiomyocytes (72-96h), while adjacent uninfected cells displayed an intense network. Confocal microscopy analysis also revealed alterations in LN distribution in later times of infection. Our preliminary data demonstrated that treatment of the cultures with TGF- $\beta$  lead to a general enhancement in FN matrix, which became more interconnected and exhibited thick deposits around adjacent cells. Nonetheless, the cells displaying high number of intracellular parasites reacted weakly to TGF- $\beta$  stimulation, showing a reduced FN expression when compared to neighboring uninfected cells. Altogether, these results indicate that the enhancement of FN expression occurring *in vivo* (Andrade et al., 1989) may be an effect of TGF- $\beta$  secretion by inflammatory

cells. The differential FN expression displayed by infected cardiomyocytes *in vitro*, even after TGF- $\beta$  treatment, may be related to cytoskeletal alterations already reported in infected cardiomyocytes (Pereira et al., 1993) or low expression of integrins or TGF- $\beta$  receptors induced by *T. cruzi* infection.

References: Andrade SG et al 1989. *Am J Trop Med Hyg* 40:252; Araújo-Jorge TC et al 2002. *J Infect Dis* 186(12): 1823; Pereira et al., 1993. *J Submicrosc. Cytol. Pathol.* 25(4): 559; Rossi MA, 2001 *Med Sci Monit* 7(4): 820

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#### BC29 - *TRYPANOSOMA CRUZI* INFECTION ALTERS FOCAL ADHESION PROTEINS DISTRIBUTION IN CARDIOMYOCYTES

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Focal adhesion proteins play an important role in the contractile transmission force to the extracellular matrix during the contraction-relaxation process. Alterations in the contractibility and cytoskeleton organization have been demonstrated in Chagasic cardiomyopathy (De Carvalho et al., 1994; Fernandez et al., 1992, Pereira et al., 1993). However, the integrity of focal adhesion proteins in this cardiomyopathy is unknown. Our goal was to evaluate the focal adhesion expression during *Trypanosoma cruzi*-cardiomyocyte infection.

Indirect immunofluorescence was performed to investigate the distribution of focal adhesion proteins in uninfected and *T. cruzi*-infected mice heart cryosections and cardiomyocytes cultures. The samples were fixed with 4% paraformaldehyde in PBS. Thereafter, the cells were washed and incubated overnight at 4°C with anti-vinculin or anti- $\alpha$ -actinin antibody. The antigen-antibody complex was revealed with the appropriated secondary antibody. Controls were performed in the absence of the primary antibody.

In the health myocardium, vinculin was detected in striated pattern called costameres and also in the intercalate disk of cardiomyocytes. Preliminary data revealed a similar distribution of vinculin in the infected tissue during the acute experimental Chagas' disease. The immunolocalization of vinculin in cultured cardiomyocytes revealed its distribution anchoring the myofibrils to sarcolemma, co-localized at the Z-line (costameres) and also localized at the cell-substrate adhesion sites, while  $\alpha$ -actinin was visualized in the Z-line and similarly associated to the cell-substrate adhesion sites. Both focal adhesion proteins, vinculin and  $\alpha$ -actinin, displayed changes in the striation pattern in *T. cruzi*-infected cardiomyocytes. These data suggest that alterations reported in the contraction mechanism may also be related to the disturbance in the localization of focal adhesion proteins.

References:

De Carvalho et al., 1994. *J Cardiovasc Electrophysiol.* 5(8):686; Fernandez et al., 1992. *Acta Physiol Pharmacol Ther Latinoam.* 42(3):197; Pereira et al., 1993. *Submicrosc. Cytol Pathol* 25(4): 559

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#### BC31 - ANALYSIS ON THE FUNCTION OF MANNOSE-FUCOSE RECEPTOR IN THE UPTAKE OF *LEISHMANIA (L.) AMAZONENSIS* BY MICROGLIOCYTES IN GLIAL MIXED CULTURES

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Microglia are resident macrophages of the brain, which normally are quiescent or down-regulated when compared to other tissue macrophages. However, in chronic neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and prion disease they are activated and express molecules not seen on the normal resting microglia. In recent years, there is much interest in studying the interaction of microglia cells with protozoa. *Leishmania* are pathogenic obligatory intracellular parasites, which must gain entrance into mononuclear phagocytes to successfully complete their cell cycle. Actually is well known that receptors interacting with terminal sugars as ligands are involved in the binding of *Leishmania* promastigotes to the macrophage surface and their subsequent internalization. In the present study, we have evaluated the involvement of the Mannose-Fucose Receptor (MFR) in the uptake of *Leishmania* by microglial cells in mixed glial cultures.

Microglia primary cultures were obtained from newborn mice brains and the cells were dissociated by successive cycles of mild incubation with trypsin. The cell suspension was plated and maintained at 37°C in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. For *in vitro* modulation of MFR, the cultures were pre-incubated for 24h with 0.1 mg/ml DM or 1 mg/ml LPS. Thereafter, the cultures were maintained for 2 h at 37°C in the presence of *L.(L.) amazonensis* promastigote forms in a 10:1 ratio. After this period, cultures were rinsed with PBS to remove extracellular parasites and fixed after 0, 2, 6, 12, 24, 48 and 72h. Infected cells were fixed with Bouin's solution, stained with Giemsa and observed by light microscopy.

To analyze the expression of MFR during the infection periods, the infected microglial cells were labeled with the neoglycoprotein, mannosyl bovine serum albumin (mannose-BSA) coupled to FITC and their morphology was compared with DM-treated cultures and non-infected cultures. The samples were then fixed for 5 min with 4% paraformaldehyde (PFA) at room temperature and incubated with mannose-BSA for 1h at 37°C.

Our preliminary results showed that all types of microglia (ramified and ameboid) expressed the MFR on their surface, with infected cells tending to display a different morphology in comparison to control and DM-treated cells. In the case of DM-treated cells, we detected punctual MFR fluorescence distributed close to the nucleus and around the parasitophorous vacuole of the host cells.

These results confirm the involvement of the MFR in the uptake of *Leishmania* by microglial cells. An indirect evidence for this fact is that addition of D+-mannose to mixed DM-treated cultures impairs both microglial infection and internalization of the mannose-BSA.

### BC32 - THE LAMININ-1 RESIDING ADHESION SEQUENCES FOR THE PARASITIC PROTOZOA *TRITRICHOMONAS FOETUS* AND *LEISHMANIA (LEISHMANIA) AMAZONENSIS*.

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The Laminins are large basement membrane matrix glycoproteins with multiple domains each one of them may determining different cell functions. The isoform 1 of Laminin (LMN-1) is known to be recognized by several pathogens including parasitic protozoa such as *Tritrichomonas foetus*. Our preliminary results showed that promastigote forms of *Leishmania (L.) amazonensis* like *T. foetus* recognized LMN-1. The Laminin-1 peptides A13, A208, AG73 (a chain), and C16 (b chain) have already been sequenced and characterized in terms of what cell functions they are preferentially involved.

Here, we designed experiments looking for which are the LMN-1 peptides preferentially recognized by the parasites *T. foetus* and the promastigote forms of *L. (L.) amazonensis*. LMN-1 (20mg.mL<sup>-1</sup>) was adsorbed onto plastic slides, and overlaid or not with each one of the four peptides. Parasites which have been coated or not with each one of the peptides were allowed to interact with the LMN-1-coated surfaces. Both *T. foetus* and *L. (L.) amazonensis* adsorbed onto LMN-1-coated surfaces and the peptides revealed to be inhibitory at different degrees when they were found recovering LMN-1-coated surfaces. These results lead us to conclude that both parasites could recognized LMN-1 through each one of the four assayed peptides. We are now searching for the cell functions determined by the peptides in the parasites, and identifying the LMN-1 surface receptors in *T. foetus* and *L. (L.) amazonensis*.

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### BC33 - *TRICHOMONAS VAGINALIS* VIRULENCE AGAINST EPITHELIAL CELLS AND MORPHOLOGIC VARIABILITY: COMPARISON BETWEEN A WELL-ESTABLISHED STRAIN AND A FRESH ISOLATE.

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The FMV1 strain of *Trichomonas vaginalis* was freshly isolated from an asymptomatic patient and its morphological properties and virulence *in vitro* compared with the well-established JT strain. The morphology variability of the parasites was assessed by differential interference microscopy and both scanning and transmission electron microscopy. The FMV1 strain presented nearly 20% ameboid cells whereas the JT strain presented high percentages of ellipsoidal cells and no ameboid ones. The FMV1 morphotype population was not altered for at least one year sub-culturing. Electron microscopy studies revealed that this strain produced numerous pseudopod structures which mediated intimate contact among trophozoites. Dead FMV1 parasites were often phagocytosed by the conspecific cells. We also compared the cytolytic capacity of these two populations against epithelial MDCK cells and its contact-dependence. The FMV1 strain rapidly adhered to plastic or glass surfaces and to MDCK monolayers. This strain destroyed about 93% of the epithelial cells in 90 min whereas the cytolytic activity of JT parasites was remarkably lower (about 41%). Parasite supernatants displayed no cytolytic activity indicating a contact-mediated lysis. The protozoan virulence *in vitro* did not correlate well with the clinical observations. The implications of these results are discussed.

Financial Support: CNPq, FAPERJ, FINEP and PRONEX.

### BC34 - DIFFERENCES IN THE NITRIC OXIDE PRODUCTION IN SPLEEN AND LIVER OF *CALOMYS CALLOSUS* INFECTED WITH *TRYPANOSOMA CRUZI*.

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Nitric oxide (NO) has a major role in macrophage defense against intracellular microorganism, including parasites such as *Trypanosoma cruzi*. On the other hand, NO is also known as a double edged sword, generated at high levels it causes intense tissue necrosis. The aim of this work was to determine the NO-production in *Calomys callosus* infected with two different strains of *T. cruzi*. Male *C. callosus* were i.p. infected with  $4 \times 10^3$  blood derived trypomastigote forms of the Bolivia (BOL, group 1) and the Boliva-sobrenadante (BOL-SB, group 2) strain. At day 5, 7, 9, 12, 14 and 45 after infection, hepatocytes and splenocytes were prepared from whole organs and cultured for 42h in appropriate medium either with LPS (1mg/ml) or without. Nitrite levels of the supernatants were measured by the Griess-reaction. Parasitemia curves were obtained at the same days after infection. Splenocytes of *C. callosus* were unable to produce NO during the infection with Bol-SB, whereas Bol infected cells showed enhanced values at day 9 and 12 (highest parasitemia) compared to controls without infection. Stimulation with LPS leads to an earlier (day 5/7) and higher production of NO, being most severe on day 14 in both infection models. In contrast, hepatocytes infected with Bol showed no significant differences during infection compared with non-infected controls. Remarkably, stimulation with LPS led to a significant decrease at day 12 (peak of parasitemia), followed by an increase to the highest values at day 14. Bol-SB strain caused a decrease of NO in hepatocytes already during an early stage after infection (day 5/7) reaching control levels at day 12 and 14. This decrease was even more pronounced after LPS stimulation. The *T. cruzi* strain Bol, with a predominance of broad structured forms, caused significant higher values of NO in splenocytes as the slender Bol-SB strain according to the different morphology and pathology described in literature, which is more severe in Bol-infected animals. In hepatocytes increased levels of NO were not observed as they were detected in splenocytes. During the course of infection with *T. cruzi* it seems that the production of NO is decreased in liver-cells. Since the NO-system has to be triggered very accurately, increased levels of NO already detected in non-infected cells compared to splenocytes, indicate that further stimulation, provoked by the parasites, may turn on other mechanism to prevent an overproduction of NO in these highly specialized metabolic cells. This could be one reason for the observed decrease in NO production during infection with two different strains of *T. cruzi*. Further experiments are carried out to analyze the triggering of NO production in this resistant animal model.

### BC35 - INFECTION OF MOUSE DERM – DERIVED FIBROBLASTS BY MONOXENOUS TRYPANOSOMATIDS – *HERPETOMONAS ROITMANI* AND *CRITHIDIA DEANEI*.

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Introduction: Flagellate protozoa of the Trypanosomatidae family, which includes agents of important parasitic diseases such as leishmaniosis and Chagas' disease, infect a wide range of hosts including animals, plants and protists (Vickerman, 1994). Monoxenous trypanosomatids are usually found in insect hosts and are considered to be not capable to cause parasitic diseases in vertebrates (Wallace, 1966). To investigate whether the trypanosomatids *C. deanei* and *H. roitmani* are able to infect vertebrate cells, we have used derm-derived mouse fibroblasts as experimental model. Skin fibroblasts were chosen as putative vertebrate hosts cells first due to some reports pointing to a role for trypanosomatids other than *Trypanosoma* and *Leishmania* in some opportunistic cutaneous infections observed in immunocompromised human individuals (Boisseau-Garsaud et al. 2000; Dedet et al. 1995), and second due to a previous report of fibroblast infection by *Leishmania (L.) amazonensis* (Corte-Real et al., 1995).

Materials and Methods: *C. deanei* and *H. roitmani* were incubated for

determined times (2 hr to 48 hr) with mouse derm-derived fibroblasts at 28 °C in DMEM medium. Thereafter, the cultures were rinsed with PBS and processed for light or electron microscopy. For light microscopy, the cells were fixed with Bouin's solution and stained with Giemsa. The percentage of infection was analysed under a Zeiss photomicroscopy.

For scanning electron microscopy (SEM), the glass coverslips containing infected fibroblasts cultures were fixed for one hour with 2.5% glutaraldehyde diluted in 0.1 M cacodylate buffer, pH 7.2, washed in buffer and then post-fixed with 1% osmium tetroxide ( $OsO_4$ ) diluted in cacodylate buffer, pH 7.2. After the coverslips were processed to be analyze in a Zeiss DSM-640 scanning electron microscopy operating at 15kV. To be observed by Transmission electron (TEM), infected fibroblasts, as well as control parasites and uninfected fibroblasts were fixed for one hour with 1% paraformaldehyde /1% glutaraldehyde diluted in 0.1 M cacodylate buffer, pH 7.2. And, then, washed and post-fixed with 1%  $OsO_4$  in cacodylate buffer. After, dehydrated in graded acetone, and embedded in PoyBed resin, ultra-thin sections were obtained in a Reichert OmU3 ultramicrotome and observed in a Zeiss EM-10C transmission electron microscope, operating at 80kV.

Results and conclusions: According to traditional protozoological concepts, monoxenous trypanosomatid protozoa do not infect vertebrate cells. We show that the monoxenous trypanosomatids *C. deanei* and *H. roitmani* are able to infect derm-derived mouse fibroblasts in vitro, as observed by light and electron microscopy. Our data are the first experimental evidence showing the phagocytosis and survival of two known monoxenous trypanosomatid parasites into vertebrate cells. We consider the possibility that *C. deanei* and *H. roitmani*, and perhaps other monoxenous trypanosomatid species, can cause opportunistic infections in immunocompromised individuals and possibly cause cutaneous lesions in vertebrate hosts.

Financial support: FAPERJ/ FIOCRUZ.

### BC36 - CHEMOKINES AND INFLAMMATION DURING EARLY RESPONSE TO *L. (V.) BRAZILIENSIS* INFECTION.

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In this study, we investigated the early immune response of *L. (V.) braziliensis* isolates that display different biological behavior in BALB/c mice, in order to understand the early inflammatory events and the pathogenesis of *L. (V.) braziliensis* infection. Of utmost interest, we want to establish whether the development of cutaneous leishmaniasis due to different *L. (V.) braziliensis* isolates can be associated with a specific chemokine profile and inflammatory response. Mice were infected with two *L. (V.) braziliensis* (CE-3227 and BA-788) and disease evolution was determined through weekly measurement of lesion sizes and assesment of parasite load. Cytokines, chemokines and chemokine receptors mRNA expression in footpad lesions was evaluated at various days post infection. The results shown that the infection with CE-3227 always resulted in larger lesions than that with BA-788 (0.129 0.013 vs 0.085 0.006). CE-3227 isolate seems to induce a more intense inflammatory reaction than BA-788, since parasite burden did not correlate with lesion development in the inoculation site. IFN- $\gamma$  mRNA was detected earlier in BA788-infected mice than in CE3227-infected mice while IL-4 mRNA was observed at 3 days post infection with both isolates. The level of the chemokines and chemokines receptors expression in lesion tissue of infected mice was markedly different between the two parasite isolates. CE-3227 isolate upregulated the expression of more chemokines and chemokines receptors than BA-788 mice. Of interest, the expression of CCL2/JE/MCP-1, CCL3/MIP-1 and their receptors was more

strongly induced in CE3227-infected mice than in BA788-infected one. These findings suggest that differences in virulence presented by *L. (V.) braziliensis* isolates can induce specific chemokine expression at the inoculation site, which would implicate in immunological effects in the early inflammatory events and development of the differentiated pathogenesis in the *L. (V.) braziliensis* infection at a later time.

This work was supported by PROCAD/CAPES/FAPESB.

### BC37 - PRELIMINARY SURVEY OF FREE-LIVING PROTOZOAN SPECIES FROM THE FURNAS LAKE (MG, BRAZIL): MORPHOLOGICAL AND MOLECULAR APPROACHES.

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The Furnas lake is an important artificial reservoir located in the south of Minas Gerais, Brazil. It plays a significant role in human activities as recreation, economical interest and human health. Free-living protozoa have a large distribution and a very significant role in aquatic microecosystems as they take part in a complex food web including all trofic levels, thus collaborating for nutrient recycling.

To study the protozoa species from the lake, water samples from 3 points of the Furnas lakes near the city of Alfenas were collected with plankton nets and bottles. The samples were examined one or two days later. The organisms were observed *in vivo* under a stereoscopic microscope where each organism could be isolated using glass micropipettes. In order to have a large number of organisms of each species for identification, different media were tested. The most successful ones were 997 (ATCC Bank) and a medium enriched with rice. For each species different fixative agents and some staining methods were tested in order to identify them by means of morphological characters useful for taxonomy. For a flagellate species we have also performed Normanski differential interference contrast microscopy.

Molecular techniques using DNA extraction and PCR reactions with primers specific for the mini exon donor RNA (medRNA) were used for the identification of an organism from the Bodonidae family.

In the present study we have found three great groups of protozoa in the Furnas lake region examined: ciliates (the most frequently and the group that shows the major diversity of morphospecies), flagellates and amoeba species. For the ciliates, species identification was done after silver impregnation technique by Dieckmann (1995) and Fernandez-Galeano (1976), revealing somatic and oral infraciliature and the nucleus. These characteristics lead us to identify 9 genus of ciliates by comparison with key identification and figures (Silva & Silva-Neto, 2001; Sleigh, 1989; Sorokin, 1999). For the flagellates, 3 genus could be identified, including a *Bodo* species, confirmed by molecular analysis that shows a PCR product of ~450bp using primers specific for the medRNA, whose size was already described for this genus by Santana *et al.* (2001).

Our preliminary results clearly shows that water samples from Furnas lake exhibit a large diversity of protozoa morphospecies, all of them already described and commonly found in freshwater samples and sewerage discharge (Silva & Silva-Neto, 2001; Sleigh, 1989). Our work also described for the first time the isolation of a *Bodo* species in an artificial reservoir, with its identity confirmed by molecular analysis.

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### BC38 - DIMORPHISM IN *TRYPANOSOMA CRUZI*: A COMPARATIVE STUDY OF DISTINCT STRAINS

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One of the unsolved aspects of the cell biology of the *Trypanosoma cruzi* is the occurrence of slender and broad forms. Several studies were dedicated to explain the biological reason of the dimorphism in *T. cruzi*. They analyzed the following aspects concerning the presence of slender and broad forms: a) appearance and persistence in the bloodstream; b) infectivity to mammals and vectors; c) prevalence of each form in different strains; d) tissue tropism; e) motility and; f) behavior in tissue culture. In the present study a comparative analysis of the morphology, motility and cell surface antigenic composition of slender and broad forms were done. Trypomastigotes forms of the Y strain and CL-Brener and Dm28c clones were used. To analyze the influence of the cell size on the number of trypomastigotes produced at the end parasite cycle and the mechanical resistance of the cell to the movement of trypomastigote forms LLC-MK<sub>2</sub> cells were used. The first free trypomastigotes forms were harvested from the culture medium after 5-6, 6-7 and 3-4 days post-infection with the Y strain and CL-Brener and Dm28c clones, respectively. Preliminary studies using of the Dm28c clone showed that the length of trypomastigotes liberated from day 4-8 post-infection tends to increase whereas the width tends to reduce. The mean length of Dm28c trypomastigotes obtained after the first liberation (4<sup>th</sup> day post-infection) is  $10.7 \pm 2.9\mu\text{m}$  and in the 8<sup>th</sup> day post-infection is  $15.2 \pm 3.4\mu\text{m}$ . The width varies from  $1.6 \pm 0.4\mu\text{m}$  to  $1.2 \pm 0.2\mu\text{m}$  in the 4<sup>th</sup> and 8<sup>th</sup> days post-infection, respectively. Analyses of the Y strain and CL-Brener clone are in progress. Preliminary data have shown that Y strain is mainly composed by thin trypomastigotes presenting a mean of  $19.1 \pm 3.1\mu\text{m}$  in length and  $1.0 \pm 0.2\mu\text{m}$  in width. Broad forms present  $12.9 \pm 2.3\mu\text{m}$  in length and  $1.4 \pm 0.2\mu\text{m}$  in width. Thin trypomastigotes of CL-Brener clone are  $17.9 \pm 2.3\mu\text{m}$  long and  $1.0 \pm 0.2\mu\text{m}$  in width, whereas broad form are  $11.9 \pm 2.4\mu\text{m}$  and  $1.6 \pm 0.4\mu\text{m}$  in length and width, respectively. Thin forms of Dm28c clone trypomastigotes present  $15.9 \pm 2.1\mu\text{m}$  in length and  $2.1 \pm 0.2\mu\text{m}$  in width whereas broad forms are  $8.3 \pm 1.0\mu\text{m}$  and  $1.4 \pm 0.2\mu\text{m}$  in length and width, respectively. Immunocytochemical analysis of the cell surface of slender and broad forms using antibodies against SAPA, 80kDa, LPPG and a-Gal showed that the broad forms of the different samples were always more intensely labeled than slender ones. Comparisons about the velocity of displacement and kind of movement on slender and broad forms of the three samples, using video-microscopy are in course.

Supported by: CNPq, CAPES, FUJB/UFRJ

### BC39 - FURTHER ULTRASTRUCTURAL STUDY ON THE ENDOMASTIGOTE FORM OF A TRYPANOSOMATID ISOLATED FROM SOLANACEAE

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We have previously reported on the isolation and cloning of a trypanosomatid from a tomato fruit which presented a endomastigote form (Coelho *et al.*, *Rev. Inst. Med. Trop. S. Paulo*, 44 Suppl.: 97, 2002). Here we report on a initial ultrastructural analysis of this isolated based on transmission electron microscopy (TEM). Cells were fixed for 2 h with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2, containing 50 mM CaCl<sub>2</sub>. The cells were then

washed in the same buffer and postfixed for 1 h at room temperature in a 1% OsO<sub>4</sub> solution in 0.1 M cacodylate buffer, pH 7.2, containing 0.8% potassium ferricyanide. After postfixation, cells were dehydrated in acetone and embedded in Epon. Ultrathin sections were briefly stained with uranyl acetate and lead citrate and observed in a Zeiss EM-900 transmission electron microscope operating at 80 kV. Results showed cells with a long curved flagellar pocket, characteristic of the endomastigote stage of the *Wallaceina* genus (Podlipaev et al., Parazitologia, 30: 324-332, 1990, in Russian). However, some distinctions were also observed as a lack of a double set of four microtubules in the area of the flagellar pocket, and a presence of a large mitochondrion. Previous molecular analysis showed that *Leptomonas peterhoffi* and *Blastocrithidia gerricola* are close related with *Wallaceina brevicula* and *Wallaceina inconstans*, though they also show morphological differences. Thus, we conclude that the presence of the endomastigote stage strongly suggests that the isolated from Solanaceae may belong to the *Wallaceina* genus but biochemical and molecular analysis will be required to better characterize this trypanosomatid.

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#### BC41 - HUMAN STERILE URINE AS *LEISHMANIA* ENRICHMENT FACTOR FOR PRIMARY GROWING. INFLUENCE OF DIFFERENT SOURCES OF URINE AND THE COLLECT BIOLOGICAL SAMPLES FROM DOGS NATURALLY INFECTED IN KALAZAR ENDEMIC REGION.

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The aim of this study were investigate the importance of: a. the source of human urine to be used as enrichment factor of growing *Leishmania* in acellular culture media without fetal serum calf; b. the procedure for collecting biological samples from naturally infected dogs in canine kalazar endemic region.

Human urine collected from four different donors, a 47 years old man; a 40 years old woman; a 8 years old boy; a 6 years old girl, were sterilized by filtration in 0,22µ filter.

Four groups of 16x160 tubes of biphasic media (blood agar base as solid phase and brain heart infusion as liquid phase) were constituted. Each group was supplemented, respectively, with 5% of different human sterile urine described above, calculated to the final concentration of the liquid phase.

Spleen aspirate was obtained from 44 dogs with clinical signs of canine visceral leishmaniasis. All dogs examined were from Araçatuba region in São Paulo State most of them presented previous confirmed positive diagnosis. Two procedures were utilized in order to collect spleen aspirate: a. vacuum aspiratory system that turned possible direct inoculation in medium culture tube working in field condition; b. aspirate transferred to a recipient containing antibiotic saline solution and the inoculation into culture tube was did under laboratory condition in a sterile flow cabinet.

Vacuum aspiratory system permitted to us isolate *Leishmania* from 90% of infected dogs, while the second procedure maked possible isolation from 70% of the same animals. Qui square test showed no statistical significance for observed differences.

In previous studies we demonstrated that human sterile urine could optimize 2-3 times more *Leishmania* isolation than without any supplementation (Rev. Inst. Med. trop. S. Paulo, 44:2002; Rev. Soc. Bras. Med. Trop., 36 :2003). In this study we obtained again the high performance of *Leishmania* isolation when human sterile urine was utilized as growing factor, but no statistical differences were obtained, by qui square test, when considered the source of urine, for either different sex or age.

Again we concluded that the advantages of human urine utilization, are low

costs and excellent technical performance, applied also for vacuum system, as alternative for *Leishmania* isolation in field conditions.

#### BC42 - GAMMA IRRADIATION AS A TOOL FOR METACYCLIC *LEISHMANIA (L.) AMAZONENSIS* SELECTION

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*Leishmania* spp. causes a spectrum of human diseases, ranging from self-healing skin lesions to severe and lethal visceral disease. The parasites are transmitted sand fly bites, and have two morphologically distinct forms on its life cycle: promastigotes, a flagellated that occurs in the insect gut, and amastigote form, an small obligatory intracellular parasite. In previous work we demonstrated that the protein and nucleic acid metabolism and oxidative respiration were severely affected by irradiation, in a dose response way, but a small but representative fractions are relatively radio resistant, surviving after 800 Gy of <sup>60</sup>Co irradiation. The best explanation could be a selection of metacyclic promastigotes. In these forms, the Go state allows the adequate correction of DNA repair after the irradiation insult. In this work, we are looking for the ideal radiation dose to select the higher proportion of metacyclic forms of *L. (L.) amazonensis* in culture. Parasites were grown in RPMI 1640 medium, plus 20% fetal calf serum, at 24° C, in monophasic medium until log stage were achieved. For the determination of metacyclic forms, we used the infection of mammalian cells, RAW (macrophage-like) cells, aside to the classical complement resistant behavior of the metacyclic form. Parasites were cultured for 7 days and their growth determined in Neubauer chambers. Initially, the parasites grow exponentially, procyclic forms, until the fourth day, while on the fiftieth and sixth we observed a declining growth that disappears at the 7<sup>th</sup> day, when the *Leishmania* numbers became stable or reduced. Those forms were recovered by centrifugation, suspended in fresh TC-100 medium without serum, adjusted to 10<sup>7</sup>/ml promastigotes and irradiated with doses ranging between 25 and 400 Gy of <sup>60</sup>Co gamma-radiation. The number of surviving intact parasites was counted in hematocytometer chamber. As expected, there is a dose response effect on parasite numbers, with 400 Gy shown the lowest number of parasites. Irradiated promastigotes were challenged to 2x10<sup>5</sup>/ml RAW cells in round cover slips, in RPMI 1640 medium, plus 10% fetal calf serum, at 37°C 5%CO<sub>2</sub> in 24-well plates by 3 hs. The cover slips were fixed, stained with Giemsa, with determination of the number of infected cells. Parasites irradiated at 400 Gy infected, proportionally, more cells than parasites irradiated at other doses. To confirm this metacyclogenesis, a complement lysis assay was performed with 5, 10 and 20% of male guinea pig blood serum at 20°C for 3 hours, and parasites counted. Guinea pig serum a 10% promotes more lysis, with 200 Gy irradiated parasites being less affected, probably due to metacyclic selection. These preliminary results suggests that the ionizing radiation, specially between 200 and 400 Gy, could be a alternative tool for the selection of metacyclic forms of *Leishmania (L.) amazonensis* in culture.

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#### BC43 - SURAMIN TREATMENT LEADS TO FLAGELLAR DETACHMENT IN *TRYPANOSOMA CRUZI* TRYPOMASTIGOTES

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Suramin, a symmetrical polysulfonated naphthylamine derivative of urea, is a potent antagonist to some P2 receptors and is able to inhibit a large number of cellular enzymes, such as ecto-ATPases, retrovirus reverse transcriptase, DNA polymerase, among others. It had initially been used in sleeping sickness treatment and is under study for therapeutic treatment of some cancers. Morphological changes such as plasma membrane disruption of chick embryo neural retina, axonal degeneration or atrophy, which may lead to neuronal apoptotic cell death, alterations on distribution of actin filaments in epithelial cells and inhibition on formation of stress fibers and focal contacts in endothelial cells, which may interfere on cellular migration were observed after treatment with suramin. In this study we analyzed suramin effects on *T. cruzi* trypomastigotes flagellar adhesion to cell body. Y strain parasites were obtained from the supernatant of infected LLC-MK<sub>2</sub> cells cultivated in RPMI-1640 medium supplemented with 2% of fetal calf serum. 500 µM suramin were added to culture medium 24 h after infection and was maintained during *T. cruzi* intracellular cycle. Trypomastigotes cultivated in the presence of suramin are about 20% shorter and about 25% broader when compared to control cells and present parts of the flagellum or the whole flagellum detached from cell body. It was observed by videomicroscopy that suramin treated trypomastigotes present cellular movements about 3-fold slower when compared to control cells. In preliminary studies we observed, in drug treated trypomastigotes with the flagellum detached from cell body, an intense labeling both in cellular body and in the flagellum when we used the polyclonal antibody, for the repetitive flagellar antigen (FRA), which is an antigen located on the side of epimastigotes and trypomastigotes flagella that faces parasite body. This result was different from that observed with control cells, which present an intense labeling in the flagellum and just a weak labeling in cell body. Preliminary results show that suramin addition to culture medium during *T. cruzi* intracellular cycle may affect parasite division, since the presence of two flagella in some treated trypomastigotes was observed. This result probably is due to a disorganization on the arrangement of internal flagellar attachment zone structures in cells with the flagellum detached from cell body, since the correct organization on the arrangement of these structures is essential for the cell shape and division plane definition.

Supported by: CNPq, CAPES, FUJB/UFRJ

### BC44 - ANALYSIS OF THE CYTOTOXICITY OF MNNG ON THE PROLIFERATION OF *HERPETOMONAS SAMUELPESSOAI* USING THE MTT ASSAY

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*Herpetomonas samuelpeessoai* corresponds to a Trypanosomatidae parasite from the *Herpetomonas* genus characterized by three different differentiation forms, promastigota, paramastigota, and opisthomastigota. These parasites can be grown in culture medium *in vitro*. Morphological, biochemical, growth, differentiation and motility alterations can be observed in these organisms after treatment with various drugs. MNNG (N-methyl N-nitro N-nitro guanidine) is an established carcinogenic substance with confirmed *in vivo* neoplastic action, and also with cytotoxic and mutagenic effects on *in vitro* mammalian cells. MNNG was shown to be less cytotoxic and highly mutagenic in mammalian MT1 cells. The objective of this study is to evaluate the cytotoxic effect of MNNG on *Herpetomonas samuelpeessoai* growth. This effect was evaluated through the MTT assay using an automatic colorimetric method with a tetrazolium salt and spectrophotometric quantification. The inoculum was performed with 0,2 ml at the concentration of 2,4x10<sup>6</sup> cells/ml. The addition of MNNG was performed at the concentration of 10, 50, 100, 150 mg/ml. A sample without treatment was used as control. The experiments were repeated three times. The samples were incubated for 48 h at 28°C, and the parasites were thereafter submitted to the MTT assay and quantified spectrophotometrically

with an ELISA reader, followed by the evaluation of absorbance at a wavelength of 560 nm. The results from the absorbance reading demonstrated in the control group and in the samples treated with 10, 50, 100, 150 mg/ml of MNNG, the following values: 301, 30, 12, 10, and 6, respectively. The results showed that, even at lower concentrations of MNNG (10 mg/ml), there was a 90% reduction of the absorbance of *H. samuelpeessoai* reading. It can be concluded from this study, that following a 48 h incubation with MNNG, there was a reduction of the number of the parasites, demonstrating a marked cytotoxic effect of MNNG on *H. samuelpeessoai* using the MTT assay.

This work was sponsored by PROBIC-PUC MINAS.

### BC45 - DIBUCAINE INDUCES A DOWN-REGULATION OF THE CRUZIPAIN EXPRESSION IN *TRYPANOSOMA CRUZI* EPIMASTIGOTE FORMS

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Dibucaine, a local anesthetic, was found to inhibit a Ca<sup>2+</sup> independent phospholipase A2 (iPLA2), an enzyme that releases arachidonic acid (AA) from phosphatidylethanolamine. Experiments in different cell types have shown that AA is involved in membrane fusion, degranulation, secretion and phagocytosis. Previous study of our group have shown that dibucaine arrest the growth of *Trypanosoma cruzi* epimastigote forms *in vitro* and also disturb the degradation of proteins in the reservosomes, indicating a possible role of dibucaine in the inhibition of the proteolytic process that occurs inside these organelles. In the present study, the role of dibucaine in the protein and proteolytic activity expression in *T. cruzi* epimastigotes were investigated. After growth of epimastigote forms of *T. cruzi* in LIT medium in the absence and in the presence of 50 and 100 mM of dibucaine, for 48 h at 28°C, the protein and proteinase contents were analyzed on SDS-PAGE and Western-blotting. Epimastigotes grown in the presence of both dibucaine concentrations, showed qualitative changes in the protein profile with marked preferential expression of polypeptides of 95 and 56 kDa when compared with non treated cells. Gelatin-SDS-PAGE analysis revealed two major proteolytic activities in epimastigotes of *T. cruzi*, when incubated in acidic pH: a 40 kDa cysteine proteinase (cruzipain activity), inhibited by 10 mM E-64, and a 52 kDa metalloproteinase, restrained by 1 mM 1,10-phenantroline. The parasites cultivated in the presence of 50 and 100 mM of dibucaine demonstrated a significant reduction (approximately 4-fold) in the cruzipain activity, a known virulence factor of this human pathogen. In order to characterize if the dibucaine exerts its inhibitory effect directly in the enzyme structure by producing conformational changes, non-treated epimastigote cellular extract were incubated with distinct concentrations of the drug (50, 100, 250 and 500 mM) for 1 h and 4 h. In this case no reduction in cruzipain activity was observed. Moreover, no alteration in the proteolytic activity was observed when samples were separated by gelatin-SDS-PAGE and incubated for 20 h at 37°C, in digestion buffer (pH 5,5) supplemented with 100 mM of dibucaine. Therefore, we hypothesized that dibucaine might be influencing the cruzipain synthesis, once this drug interferes with signal transduction pathways. Western-blotting analysis showed a dramatic reduction in the cruzipain polypeptide synthesis in dibucaine-treated parasites in comparison with non-treated ones. Here, we demonstrated that dibucaine inhibits the major cysteine proteinase activity of *T. cruzi*.

Supported by: CNPq, CAPES, FUJB-UFRJ.



**BC46 - EVIDENCE FOR NITRIC OXIDE ROLE IN *T. CRUZI*-TRIGGERED NEURONAL LESIONS *IN VITRO***Almeida-Leite C.M.\*<sup>1</sup>, Galvão L.M.C.\*\*<sup>2</sup>, Cunha F.Q.\*\*<sup>3</sup>, Arantes R.M.E.\*

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Objectives: Neuronal lesion and peripheric denervation in Chagas' disease are related to local inflammation which is triggered by *T. cruzi* infected macrophages and activated lymphocytes. Although the human disease is associated with autonomic and enteric nervous system lesions, the pathogenic mechanisms of neuronal lesions in the heart and megavisceras are still unclear. We have previously developed an *in vitro* co-culture model of sympathetic cervical ganglion (SCG) neurons and macrophages (Arantes et al, 2000) and we have showed the role of macrophages in reducing *in vitro* neuronal survival average to 70%. At the present work, we investigate the interaction of nitric oxide production, neuronal lesion and survival in *T. cruzi* infected neurons and IFN- $\gamma$  activated macrophage co-cultures. Methods: Primary neuron cultures of SCG neurons were prepared as described previously (Blennerhasset & Bienenstock, 1998). Briefly, after the removal of C57BL/6 newborn mice ganglia, the neurons were isolated by enzymatic and mechanic dissociation. The cells were then cultured on Matrigel (Becton Dickinson, NJ) and 50ng/ml of NGF and were maintained for 48 hours before co-cultured with macrophages and infected with Y strain of *T. cruzi*, in the presence or absence of IFN- $\gamma$ .

Neuronal survival was assessed as the percentage of surviving neurons. Results: *T. cruzi* infected pure neuronal cultures didn't produce nitric oxide (NO) and didn't show reduction on neuronal survival when compared to control. Co-cultures of neurons and macrophages showed 77% of neuronal survival according to previous results, and the NO production of these co-cultures was not relevant. Infected co-cultures of neurons and IFN- $\gamma$  macrophages showed 44% of neuronal survival associated with increase of NO production. Conclusion: We believe that *T. cruzi* infected pure neuron cultures do not produce NO. The addition of IFN- $\gamma$  to infected co-cultures is related to increased NO production by macrophages and significant neuronal death which suggests a role for macrophage derived NO in neuronal lesions.

Support: UNDP/WHO Special Programme for Research and Training in Tropical Diseases, FAPEMIG (5599), \*<sup>1</sup> Master student financed by CAPES.

**BC47 - MAST CELLS QUANTIFICATION AND HISTOPATHOLOGICAL ANALYSIS IN THE HEART, SPLEEN AND LIVER DURING THE EXPERIMENTAL ACUTE INFECTION WITH *TRYPANOSOMA CRUZI* IN RATS**Ferraz WP<sup>2</sup>, Azevedo AS<sup>1, 2</sup>, Oliveira MP<sup>1</sup>, Parreira GG<sup>3</sup>, Pirmez C<sup>1</sup>, Melo RCN<sup>2</sup>.

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Mast cells are multifunctional cells capable of secreting a wide variety of mediators. Following activation, these cells express mediators such as histamine, serotonin, leukotrienes and prostanoids, as well as proteases and many cytokines and chemokines, all essential to the genesis of the inflammatory response. During *Trypanosoma cruzi* infection, it has been suggested that mast cells could contribute to the control of the parasite by recognizing and killing IgG-opsonized trypomastigotes and through secretion of mediators. Increased numbers of mast cells have been demonstrated in chagasic patients with chronic disease and in experimental models. In this work, we studied the occurrence of parasitism, inflammatory processes and distribution of the mast cells in different organs

(heart, spleen and liver) during the acute experimental infection with *Trypanosoma cruzi* in rats. Female Holtzman rats (16 animals) infected with Y strain of *T. cruzi* (300.000 trypomastigotes, i.p.) and 4 controls were sacrificed at 12 and 20 days of infection. Fragments of heart (atrium and ventriculum), spleen and liver were fixed in Carnoy's fluid and embedded in glycolmethacrylate for histopathological study and quantification of mast cells. Semi serial 3- $\mu$ m-thick sections were stained with toluidine blue/basic fuchsin or toluidine blue, pH 3.0. Amastigote nests were counted in the heart (420 fields for both atrium and ventriculum) and liver and spleen (240 fields/organ), for each group. Mast cells numbers were quantified in 300 fields/group. Acute *T. cruzi* infection did not induce an increase of mast cell numbers neither in the heart nor in liver and spleen. However, a clear increase of degranulated mast cell numbers, in parallel to a diffuse mononuclear inflammatory process and high parasitism, was observed in the heart, at day 12 of infection. In the liver and spleen, mast cells were rarely observed and if so they were seen around vessels. In the heart, mast cells were scattered in the myocardium. Parasite nests were scarce in the heart, at day 20 of infection. Amastigote forms were not observed in the liver and spleen at different times of infection. Our data suggest that the acute phase of experimental infection with *T. cruzi* induce mast cell degranulation, process that seems related to the presence of high parasite load. These cells may be involved in the parasite control or even in pathogenetic mechanisms during acute Chagas disease.

Supported by IOC/FIOCRUZ/RJ and CNPq.

**BC48 - COMPARISON OF TWO RAT MODELS USED IN EXPERIMENTAL STUDIES DURING ACUTE CHAGAS DISEASE**Almeida, C.S.<sup>1</sup>; Fabrino, D.L.<sup>1</sup>; Braga, F.G.<sup>1</sup>; Pardini, M.M.<sup>1</sup>; Leon, L.L.<sup>2</sup>, Melo, R.C.N.<sup>1\*</sup>

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Different experimental models have been used in Chagas' disease studies, but most investigations are carried out in murine model and/or *in vitro*. While different lineages of mice have been compared regarding the resistance to infection, there are few studies evaluating rat models. In the present work, Holtzman and Wistar rats were inoculated with Y strain of *T. cruzi* (300,000 trypomastigotes, i.p.) and studied at days 6, 12 and 20 post-infection (4-6 animals/group) to evaluate different patterns of acute infection (parasitemia, heart parasitism and myocarditis). For each rat, 100 plastic 5-mm sections, obtained from both atria and ventricles and stained with toluidine blue – basic fuchsin, were analyzed at an interval of 70mm. In parallel, the number of peripheral blood leucocytes was evaluated. Both rat models presented a prominent parasitemia with peak at day 10. However, when the pattern of involvement of heart, a target organ of the disease, was compared, we observed a significant and dramatic difference between the models. Holtzman rats showed a higher parasitism than Wistar rats at day 6 and especially at day 12 of infection (mean  $\pm$  SE of respectively 1542.25  $\pm$  460.40 and 56.50  $\pm$  19.50 per animal). At this time, it was observed an Intense and diffuse mononuclear myocarditis in both rats. Parasitism dropped drastically in the heart of Holtzman and Wistar rats at day 20 of infection, but remained significantly higher in Wistar model. Both rats showed a significant increase of the total number of peripheral blood leukocytes compared to controls but in different days of infection. At day 6, the total number of peripheral blood leukocytes and the absolute number of blood monocytes, cells involved in the heart parasite clearance as macrophages, was higher in Wistar rats than in Holtzman ones, but the opposite was observed at day 12. At day 20, the total number of blood leukocytes showed no difference between the rats but the number of blood monocytes was higher in Holtzman rats. Our data

indicate that Holtzman rats are more susceptible to *T. cruzi* infection, showing a prominent heart involvement compared to Wistar model. In both rats, the acute disease induced a great mobilization of cells from the monocytic lineage, but this response was more intense in Holtzman rats. In addition, Holtzman rats showed a more efficient resolution of the acute infection compared to Wistar. In spite of both rat models being able to respond to infection, our data highlight the Holtzman rat as a more reliable model for *in vivo* experimental Chagas disease studies, especially those ones focused on the monocyte/macrophage system involvement.

Supported by FAPEMIG/ FIOCRUZ/CNPq.

### BC49 - ADULT RAT INFECTIONS WITH JG STRAIN OR CL-BRENER CLONE: PARASITEMIA, HISTOPATHOLOGY AND CYTOKINE SERUM LEVELS

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The juvenile Holtzman rat has been used in our laboratory as a model for Chagas disease acute and chronic indeterminate phases, the heart being the main studied organ. It was demonstrated that the juvenile rat susceptibility depends on *T. cruzi* population. Six different populations have been tested and two of them present opposite virulence and pathogenicity, CL-Brener clone and JG strain. JG strain provokes low parasitemia, null mortality and focal myocarditis that is maintained for several months with very low if any cardiac sympathetic denervation. CL-Brener clone causes higher parasitemia with high mortality and severe myocarditis accompanied by sympathetic denervation, both of which are resolved in acute phase-surviving animals. Adult rats are known to be resistant to *T. cruzi* infection. However, despite the null or very low parasitemia, Y strain-infected adult rats develop acute myocarditis with sympathetic denervation followed by recovery. Aiming at having adult rat models for the indeterminate chronic phase of Chagas disease, we now compared JG and CL-Brener clone infections. Two-month-old rats were inoculated with 10,000 trypomastigotes/50g body weight, and sacrificed at days 5, 10, 15, 20, 37 or 120 post-inoculation. Parasitemia was very low in both infections, the values remaining below 50 parasites/5 µl of blood. Mortality was null even in CL-Brener clone infection that kills 100% of juvenile rat after inoculation of 10,000 parasites. No histological alterations were found in the brain, esophagus and rectum. Skeletal muscle (diaphragm) presented amastigotes nests only in CL-Brener clone-infected animals during the acute phase. The heart was affected by both infections. However, the JG strain-induced acute myocarditis was predominantly focal (mild or moderate) and mild focal myocarditis persisted during the chronic phase. In contrast, CL-Brener clone-induced acute myocarditis was diffuse and severe at day 15, but normality occurred at day 120. Cardiac sympathetic denervation was observed only in CL-Brener clone-infected rats at day 20 of infection (glyoxylic acid-induced histofluorescence). TNF- $\alpha$  serum levels (ELISA) increased significantly during the acute phase in both infections. However, at day 120, only in JG strain-infected animals the levels remained higher than control values. In JG infection, higher serum levels of IFN- $\gamma$  occurred only at day 15 of infection. In CL-Brener clone infection, IFN- $\gamma$  levels remained elevated throughout the acute phase but the values were similar to control values at day 120. In conclusion, despite significant differences between juvenile (previous studies) and adult rats with regards to parasitemia and mortality, in adult rats the two tested populations provoked distinct myocarditis pattern as in juvenile animals. Recovery was faster in the CL-Brener clone infection in adult rats. This finding could explain the CL-Brener infection null mortality in adult rats. Cytokines levels during the infections in juvenile rats are in course.

CNPq and PRONEX

### BC50 - IMMUNOCYTOCHEMICAL STUDY OF CANINE LIVER OF DOGS NATURALLY INFECTED WITH LEISHMANIA (LEISHMANIA) CHAGASI FROM BELO HORIZONTE, MG

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Canine visceral leishmaniasis (CLV) is an endemic disease in Brazil and dogs are the principal reservoir domestic of this parasite and play a central role in the transmission cycle to humans by phlebotomine sand flies. In the New World is caused by specie *Leishmania (Leishmania) chagasi*. The aim of this study is to quantify the tissue parasitism in order to make correlations among with different clinical status of the animal. Eighty-five infected animals with positive serological exams to *Leishmania* (IFAT, Complement fixation and ELISA) were divided in four clinical groups: controls, asymptomatic, oligosymptomatic and symptomatic (weakness, cutaneous lesions, alopecia, and clinical anemia) animals. The dogs were sacrificed with lethal dose of Thionembutal 33% (1,0mL/Kg). During necropsy, small samples of liver were obtained to prepare tissue touch preparations (smears) for LDU analysis. Other liver fragments were collected and fixed in formalin buffered solution 10% for histopathological and immunohistochemical studies. The immunocytochemistry technique (streptoavidin-peroxidase method – Tafuri, et al., 2003) was carried out to determine the amastigote forms of *Leishmania* in paraffin tissue sections. For morphometrical analysis, the parasitism was analyzed with a 40x objective of an Axiolab light microscope (Zeiss). The images viewed on a computer video screen were obtained by means of the software and relayed to a computer-assisted image analysis system (Kontron Electronic/ Carl Zeiss, Germany). The results were expressed in mm<sup>2</sup>. There was no statistical differences of the parasitism load among the defined clinical animal status (asymptomatic, oligosymptomatic and symptomatic) (p>0,05).

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### BC51 - ISOLATION OF TRYPANOSOMA CRUZI STRAIN FROM SYLVATIC TRIATOMINAE

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Since 1981, the incidence of Chagas' disease decreased due to the effective control of its natural transmission in Brazil (Dias, 2000). However, considering the wide number of vectors and sylvatic reservoirs, it is necessary a constant epidemiological vigilance to keep under control the disease occurrence. In this aspect it is important the vigilance in regions where triatomine are present. In Brazil, the species *Triatoma baziliensis* and *T. pseudomaculata* can be found in the northeast, otherwise, *Panstrongylus megistus* can be found in many Brazilian areas. Another very susceptible species to *Trypanosoma cruzi* is *T. rubrovaria* (Perlowagora-Szumlewicz et al., 1988), which is widely dispersed in Rio Grande do Sul state, except in the north mountain region. As *T. rubrovaria* is in colonization process (Ruas-Neto et al. 1991), it keeps the sylvatic cycle of Chagas's disease

and it is strongly susceptible to *Trypanosoma cruzi* it was decided to study this species in details. In 2002, *T. rubrovari* was collected in six Rio Grande do Sul towns. Two *T. cruzi* strains were isolated from 5<sup>th</sup> nymphal instar of *T. rubrovaria* collected in Passo do Guapurá, Quaraí-RS. In April/2003 another bugs capture was carried out in Quaraí in order to verify the contaminated sylvatic triatomine rate and to study the characteristics of *T. cruzi* strains isolated. From 23 to 25 April/2003 during 15 hours seven people collected by active search in six points of Quaraí-RS 684 *T. rubrovaria* individuals, 53 *T. circummaculata* and 6 *Panstrongylus tupynambai*. Out of 26 *T. circummaculata* individuals examined no Trypanosomatidae forms were found. Out of 351 *T. rubrovaria* nymphs examined, 14 (3,98%) have had Trypanosomatidae forms in their intestinal contents (13 of 5<sup>th</sup> nymphal instar and one of 4<sup>th</sup> instar). Intestinal contents of 12 *T. rubrovaria* positive were inoculated in mice *Mus musculus* Swiss. Five strains were isolated in mice and culture medium LIT nominated QB I, QJ I, QJ III, QM I, QM II. These strains are in process of morphological and molecular characterization. In this field work six nymphs of *P. tupynambai* were also collected in the same ecotope of *T. rubrovaria* as well as a wizard *Tupinambis merinae*.

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#### BC52 - OBSERVATIONS ON A NATURAL INFECTION OF MONOXENOUS TRYPANOSOMATIDS IN CALIPHORIDAE

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Insect trypanosomatids comprise a large number of species mostly founded in the orders Hemiptera and Diptera, where about 300 species of trypanomatids have been described. Several aspects of the host-parasite association have been studied in insects naturally infected with trypanosomatids but only in a few cases investigations involved monoxenous species. The present study reports, on light and electron microscopy, observations of flies (Caliphoridae) naturally infected with trypanosomatids. Forty wild flies were collected in the vicinity of Alfenas city, Minas Gerais, Brazil. The dissection of the insects was carried out on sterile saline, and the presence of flagellates was detected by phase-contrast microscopy. Infected intestines segments were fixed in paraformaldehyde 0.4% (W/V), and embedded in Histo-resin (Leica). Semithin sections were stained with basic fuchsin 0.1% for 30 min, and toluidine blue 0.1% (W/V) in borax 1% (W/V) for 15 min. For scanning electron microscopy the guts were fixed in a solution containing 2.5% (V/V) glutaraldehyde and cacodylate buffer 0.1 M, pH 7.2, post-fixed in 1% (W/V) osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, dehydrated in ethanol series 50 to 100% (V/V), dried by the CO<sub>2</sub> critical point method, and sputtered with gold. Observations and micrographies were made with a Jeol JSM-5310. Up to 70% of the flies were founded parasited by trypanosomatids. Elongated flagellated cells were mostly observed as free parasites in the endoperitrophic space, always near the peritrophic membrane, but never attached to it. Few parasites were observed in the ectoperitrophic space as well as in the gut. However in the former region, some parasites were seen putting on the flagella between epithelial cells. The infection was also detected in the region of the rectum where flagellates could be seen lined with a thick cuticular layer. Ectoperitrophic infection was only observed when the region of the rectum was founded parasited. No parasites were seen attached to the epithelial cells of the rectal pads, although the infection was always observed near them. The morphology of the parasites attached to the cuticula layer was markedly distinct from those described in the endoperitrophic space since rounded forms predominated in the rectum region. A great number of cells observed by SEM showed a protusion in the end of the flagellum including non-attached cells.

Supported by FINEP and CNPq (Pronex)

#### BC53 - DYNAMICS OF TRYPANOSOMA CRUZI CIRCULATION IN NATURAL FORESTED ENVIRONMENT IN SÃO SEBASTIÃO ISLAND (ILHABELA), NORTH SHORE OF SÃO PAULO STATE, BRAZIL.

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Since 1997 we have studied the ecoepidemiology of *Leishmania* in natural forested areas in São Sebastião Island (Ilhabela), north shore of São Paulo State. Secondly we have had opportunity to observe circulation of *Trypanosoma cruzi* like in this environment.

Between October 2000 and November 2002, monthly, we have caught wild animals in order to identify natural infection and isolate *T. cruzi*. After made identification and ear sign (a code built with a hole in the ear), for each animal, xenodiagnosis it was performed to attend this purpose. Then the animal was turned free in the same place of capture. Until now, from 551 captures, we had caught 298 different mammals (162 Marsupialia, being 139 *Philander opossum*, 22 *Didelphis aurita* and 1 *Gracilinamus agilis*; 136 Rodentia, being 122 *Proechimys iheringi*, 8 *Oxymycterus incanus*, 5 *Cavia aperea* and 1 *Nelomys thomasi*). The others 253 captures corresponding recaptures of several animals caught, marked and released before. In each recapture a new xenodiagnosis was done.

The results revealed as high is the circulation of this protozoan group, from 13.7% (19 out 139) *P. opossum*, 17.2% (21 out 122) *P. iheringi*, 4.5% (1 out 22) *D. aurita* and 25% (1 out 8) *O. incanus*, *T. cruzi* like was isolated. It is interesting to report that parasitemia was not constant. Some times several infected animals showed their positivity and some times not.

Even though the rates of parasite circulation in natural environment have been as high as we observed, a great number of animals rested still negative after 6, 12 and until 20 months and with 2 to 12 captures/recaptures.

It is clear to us that the geographic dispersion of parasite depends on the environment where the vertebrate and/or invertebrate host relationship occurs.

#### BC54 - COMPARATIVE EVALUATION OF THE INFECTIVITY AND DEVELOPMENT PATTERN OF GFP-EXPRESSING TRYPANOSOMA RANGELI IN RHODNIUS DOMESTICUS AND MICE

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*Trypanosoma rangeli* is a protozoan parasite transmitted in both Central and South America by triatomine vectors among a variety of mammal species, including humans, acting as a complicating factor for Chagas disease diagnosis. Aiming the study of the parasite life cycle, in this study we have characterized parasites of *T. rangeli* SC-58 strain transfected with the pTEX-GFPmut/SSU1rRNA plasmid by comparison with non-transfected parasites concerning their infectivity and evolution in triatomine bugs. For that, two groups of Swiss mice were infected by i.p. inoculation of 2.5x10<sup>6</sup> trypomastigotes/mice with transfected or non-transfected parasites of *T. rangeli* SC-58 strain. The infection course was evaluated daily by both fresh and Giemsa stained smears, observing the maintenance of GFP expression by transfected parasites. Positive mice were used for feeding 2 groups of 20 4<sup>th</sup>-5<sup>th</sup> instar nymphs of *Rhodnius domesticus* which were searched for the presence of parasites after 30 days of the infective blood meal by microscopic evaluation of the hemolymph, salivary glands, feces or digestive tract. Our results revealed no significant differences concerning the presence of flagellates in feces/digestive tract of *R. domesticus* infected with non-transfected (52.63%) and transfected parasites (68.42%). The infection of hemolymph and

salivary glands also not revealed significant differences between the experimental and control groups. Both transfected and non-transfected parasites were transmitted by triatomine bite, revealing patent parasitemia in Swiss mice. Quantitation of the GFP-expression in triatomines was performed during 60 days by microscopic evaluation of the hemolymph obtained from triatomines infected by intracelomic of culture forms, showed a decreasing expression level of the GFP, which corroborates former studies *in vivo*. Our results confirm the maintenance of biological characteristics of GFP-expressing *T. rangeli* as well as the reduction of the expression during the infection time course in absence of selective drug (G-418). Further studies using linearized plasmids are in progress in order to obtain stable transfections and a constitutive GFP expression.

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### BC55 - PRELIMINARY STUDY OF THE INCIDENCE OF PARASITES IN GROUPS OF RISK (STUDENTS DINERS, SALESPERSONS OF FOODS AND CONSUMPTION PRODUCTS) INSIDE AND OUTSIDE OF SAN SIMÓN UNIVERSITY IN 100 METERS RADIOUS.

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The Transmitted Illnesses for Foods (ETA) they constitute, according to the World Organization of the Health, one of the most extended problems of health in the contemporary world, and an important cause of reduction of the economic productivity (Quevedo and cabbage., 1990), because it determines a discharge morbidity rate affecting the health and quality of life (Báez and cabbage., 1993). The morbidity for intestinal parasitosis is located in third place at world level (Kancha and cabbage., 2000), the same one that is caused by contamination of the foods, being this one of the main causes of illnesses diarréicas and of bad nutrition associated to them (Motarjemi and cabbage., 1994). Some of the intestinal protozosis transmitted by more common foods that affect the man are those taken place for: *Giardia* spp., *Entamoeba histolytica*, *Entamoeba coli*, *Cryptosporidium parvum* and *Isospora* spp. that are transmitted primarily for via fecal-oral, and I eat consequence, the biggest source of contamination of foods and it dilutes it is through the pollution of these with fecal matter that contains cysts or oocysts of these parasites (Motarjemi and cabbage., 1994; Lees-Gutiérrez, 1995); this infection modality is known as infection by faeces and it happens in parasites whose evolutionary cycles are only completed in a host (Atías, 1991). One of the causes of morbidity in bolivia are the illnesses diarréicas taken place by bacterias, virus or parasites transmitted mainly by foods and it dilutes (Almanza Gutiérrez, 2002) According to data provided by the Institute Gastroenterology Bolivian Japanese of Cochabamba city in 1999 they were carried out 2072 analysis coproparasitologic, in this study 1380 (63%) t were negative and 764 (36%) were positive, of those which (11.53%) were *Giardia lamblia*. (Bustillos 2001) Therefore, the present study has as purpose to determine the presence of eggs, cysts and trofozoitos in samples of human grounds, samples of vegetables of short shaft (lettuces, carrots, radishes, turnips, spinaches), strawberries, expended in establishments of consumption public in the interior and areas inside a radius of 100 meters of San Simon University as well as students diners of the University. This study will allow us to know the existence of the problem in the student population and in the group of people that you/they elaborate and they sell foods inside and outside of the University. Through this study we will be able to know the incidence of parasites, to contribute in the diffusion of knowledge and implementation of education measures and later investigations. For the explain our objective is Istudy th the incidence of parasites in groups of risk (students diners, salespersons of foods and consumption products) inside and outside of the San Simón University and around the University in a 100 meters radious.. The examples to study come to our laboratory very early in special pockets and the

methods that we are using for the study is the direct method and the We suited at this time 1906 and have the next results in the students 82 (4,3%) for the *E. histolytica*, 4 for *Giardia lamblia* (0,21%), 1 (0,10%), 3 for *Ascaris lumbricoides* (0,21%) and *Ancylostoma duodenale* 2 (0,21%). Our study in of short shaft (lettuces, carrots, radishes, turnips, spinaches), strawberries show for us that in 20 examples, we get 1 *Balantidium coli* and lettuces we get *Ascaris lumbricoides*.

### BC56 - PHOSPHATIDYLSERINE EXTERNALIZATION AND CASPASE-3-LIKE ACTIVITY IN AMASTIGOTES OF LEISHMANIA SPP.: AN APOPTOTIC MIMICRY

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Programmed cell death, although is clearly beneficial to multicellular organisms (1), its occurrence in unicellular organisms is still a debatable issue. It has been proposed that altruistic individual death can benefit clonal population of yeasts (2) and of infective trypanosomatids (3). Amastigotes of *Leishmania* spp. are the mammalian infective forms of the parasite and they multiply inside phagolysosomes and propagate infection by sequentially infecting new macrophages when released from ruptured cells. We have shown that *Leishmania (L) amazonensis* amastigotes display phosphatidylserine (PS) at the outer leaflet of their membrane and they are able to induce inhibition of macrophage proinflammatory activity by a strategy similar to that used by apoptotic cells (4). Some of the biochemical events underlying the apoptotic phenotype are effected by members of a family of cysteine proteases displaying a mandatory requirement for cleavage after aspartic acid, collectively known as caspases (5). In the present study, we examined PS exposure and caspase-3-like activity in amastigotes of *L. (L) amazonensis* (3 different strains), *L. (L) major* and *L. (L) chagasi*. PS exposure was positive in all species above, assessed by flow cytometry using FITC-labelled annexin V as ligand; as in mammalian cells, annexin V binding to the parasites was calcium-dependent. Caspase-3-like activity in the cytosol of amastigotes was detected by a fluorimetric assay, using the synthetic fluorogenic substrate Ac-DEVD-AMC in *L. (L) amazonensis* and *L. (L) major*. Hydrolysis of capase-3 substrate (Ac-DEVD-AMC) was inhibited by caspase-3 specific inhibitor (Z-DEVD-FMK) and not by E-64 or EST, a class-specific inhibitor of cysteine-proteinases. These results seems to indicate that the apoptotic mimicry is a characteristic not only of *L. (L) amazonensis* but also others species of *Leishmania*. Apoptosis in this case, seems to be a mechanism for survival within the mammalian hosts and not for death (6,7).

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**BC57 - RNA POLYMERASE II HAS A RESTRICTED LOCALIZATION IN *TRYPANOSOMA CRUZI* NUCLEUS WHICH IS DEPENDENT ON THE TRANSCRIPTIONAL STATE**

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It has been shown that RNA polymerase II (RNA Pol II) of trypanosomes catalyzes transcription of protein encoding genes, as well as the splice leader gene, which donates the minixon attached to the 5' end of all mRNA in these organisms. Splice leader transcription has a defined promoter, while specific initiation sites are not yet recognized for most of protein encoding genes. To understand how the same enzyme transcribes both types of genes, we started to investigate the nuclear localization of the largest subunit of RNA polymerase II of *Trypanosoma cruzi* by using specific antibodies raised against a recombinant carboxy terminal domain of this enzyme. By immunofluorescence analysis, we found that the RNA Pol II labeling was not distributed homogeneously in the epimastigote nuclei. In some cells, the labeling surrounded the nucleolus region; in others, the enzyme was found in restricted areas of the nucleus. However, treatment with 20 mg/ml  $\alpha$ -amanitin, which inhibits the enzyme, completely disperses the nuclear labeling, even though we detected the same levels of the enzyme by Western blot analysis. In contrast, treatment with actinomycin D, which blocks transcription by preventing polymerase movement, promotes the appearance of several foci of RNA Pol II labeling, in addition to the major foci near the nucleolus. We conclude that large amounts of RNA Pol II are concentrated in particular nuclear domains. When the enzyme is inhibited, it seems to disperse in the nucleoplasm. But when the polymerase movement is blocked, the enzyme could be detected in many other regions. We propose that the domain found in normal conditions could be due to the transcription of highly expressed gene, probably the splice leader promoter, as the splice leader genes showed a similar localization pattern, while the foci revealed by actinomycin D treatment represent accumulation of pol II in the remaining genes.

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**BC58 - FINDING A ROLE FOR HISTONE ACETYLATION/DEACETYLATION IN *TRYPANOSOMATIDS***

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Histone acetylation has been shown to play important roles in transcription control in most eukaryotes by allowing binding of transcription factors to the chromatin. In trypanosomes, the genes encoding for histone deacetylases (HD) are present. We found that several classical inhibitors for these enzymes are unable to affect *Trypanosoma cruzi* growing, suggesting that either histone acetylation does not occur, or the enzymes involved are insensitive. To answer this question, we probed *T. cruzi* for histone acetylation. We found that histone H4 and H2a are labeled when *T. cruzi* are maintained in the presence of tritiated acetic acid and cycloheximide to inhibit *de novo* protein synthesis. No effect of trichostatin was observed. Mass spectrometry analysis showed two *T. cruzi* histone H4 species distinguished by the mass of one acetylation. Recently, histone deacetylase genes HD1 and HD3 from *Trypanosoma brucei* have been shown to be essential, and HD4 is important for progression from G2 to mitosis. Therefore, we cloned and expressed HD1, HD2 and HD4 from *T. brucei* and HD2 from *L. major* in *Escherichia coli* to study their biochemical and structural properties.

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**BC59 - IS RNAI FUNCTIONAL IN *TRYPANOSOMA CRUZI*?**

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We have previously shown that *Trypanosoma cruzi* transfected with double strand RNA (dsRNA) decreased the expression of several targets. To further confirm whether this inactivation was due to the degradation of specific mRNAs, through the process of RNA interference (RNAi), we now generated and characterized several *T. cruzi* cell lines expressing double strand RNAs. We used vectors expressing the same portion of the *trans*-sialidase (TS) gene in the sense and antisense orientation separated by 60 nucleotides. In one set of vectors the expression was driven by the ribosomal promoter, while in the other by the T7 polymerase promoter in a cell line containing this polymerase. These constructs were transfected into the parasite and stable lines selected by antibiotic resistance. We found that these stable cell lines expressed large amounts of dsRNA, which remained stable, with no decrease in the amount of TS RNA, or activity, suggesting that RNAi was not occurring. As the expressed dsRNAs were quite stable, we hypothesized that the first step in the degradation of dsRNA is absent in *T. cruzi* and therefore we tested whether transfection of small RNAs (siRNAs) was able to induce RNAi in the parasite. Thus, *in vitro* transcribed and annealed dsRNA of green fluorescent protein (GFP) was treated with DICER, generating small interfering RNAs (siRNAs), which were delivered by electroporation to epimastigote cultures of *T. cruzi* expressing GFP. Again, no decrease in GFP expression was detected. These results support the notion that *T. cruzi* is not able to perform RNAi, at least under our experimental conditions, and the previous effects might be due an anti-sense effect.

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**BC60 - HEPATIC FIBROSIS IN CANINE VISCERAL LEISHMANIASIS: A MORFOMETRICAL STUDY OF COLLAGEN FIBERS STAINED BY PICROSIRIUS RED**Patrícia da Silva OLIVEIRA<sup>2</sup>, Daniele Maria Fonseca MAIA<sup>1</sup>, Marcelo Vidigal CALIARI<sup>1</sup>, Washington Luiz TAFURI<sup>1</sup>; Wagner Luiz TAFURI<sup>1</sup>.

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Canine visceral leishmaniasis (CLV) is an endemic disease in Brazil and dogs are the principal reservoir domestic of this parasite and play a central role in the transmission cycle to humans by phlebotomine sand flies. In the New World is caused by specie *Leishmania (Leishmania) chagasi*. The aim of this study was to evaluate the parenchyma hepatic collagen deposition of dogs naturally infected with *L. chagasi*. In human beings, the hepatic fibrosis is depicted in Indian Kala-azar (Rogers et al., 1908) and in Brazil (Bogliolo, 1956). Eighty-five infected animals with positive serological exams to *Leishmania* (IFAT, Complement fixation and ELISA) were divided in four clinical groups: controls, asymptomatic, oligosintomatic and sintomatic (weakness, cutaneous lesions, alopecia, and clinical anemia) animals. The dogs were sacrificed with lethal dose of Thionembutal 33% (1,0mL/Kg). During necropsy, small samples of liver were obtained to prepare tissue touch preparations (smears). Other liver fragments were collected and fixed in formalin buffered solution 10% for histopathological and immunohistochemical studies. In all animals we observed a diffuse intralobular hepatic collagen deposition. It was characterized by Picrosirius red

staining method of all liver tissue sections under microscopic optic with polarized light. A stronger yellow-red birefringence was observed indicating one collagen type fibers deposition. For morphometrical analysis, the deposition of the collagen (fibrosis) was analyzed with a 40x objective of an Axiolab light microscope (Zeiss). The images viewed on a computer video screen were obtained by means of the software and relayed to a computer-assisted image analysis system. (Kontron Electronic/Carl Zeiss, Germany). The results were expressed in m2. There was no statistical differences among the defined clinical animals status (asymptomatic, oligosyntomatic and syntomatic) ( $p>0,05$ ). However, infected animals showed higher hepatic collagen deposition than the controls ( $p<0,05$ ). Future studies using immunocytochemistry methods for collagen will be done to confirm a type I collagen fibers deposition

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### **BC61 - FURTHER ULTRASTRUCTURAL STUDY ON THE ENDOMASTIGOTE FORM OF A TRYPANOSOMATID ISOLATED FROM SOLANACEAE**

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We have previously reported on the isolation and cloning of a trypanosomatid from a tomato fruit which presented a endomastigote form (Coelho et al., Rev. Inst. Med. Trop. S. Paulo, 44 Suppl.: 97, 2002). Here we report on a initial ultrastructural analysis of this isolated based on transmission electron microscopy (TEM). Cells were fixed for 2 h with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2, containing 50 mM CaCl<sub>2</sub>. The cells were then washed in the same buffer and postfixed for 1 h at room temperature in a 1% OsO<sub>4</sub> solution in 0.1 M cacodylate buffer, pH 7.2, containing 0.8% potassium ferricyanide. After postfixation, cells were dehydrated in acetone and embedded in Epon. Ultrathin sections were briefly stained with uranyl acetate and lead citrate and observed in a Zeiss EM-900 transmission electron microscope operating at 80 kV. Results showed cells with a long curved flagellar pocket, characteristic of the endomastigote stage of the *Wallaceina* genus (Podlipaev et al., Parazitologia, 30: 324-332, 1990, in Russian). However, some distinctions were also observed as a lack of a double set of four microtubules in the area of the flagellar pocket, and a presence of a large mitochondrion. Previous molecular analysis showed that *Leptomonas peterhoffi* and *Blastocrithidia gerricola* are close related with *Wallaceina brevicula* and *Wallaceina inconstans*, though they also show morphological differences. Thus, we conclude that the presence of the endomastigote stage strongly suggests that the isolated from Solanaceae may belong to the *Wallaceina* genus but biochemical and molecular analysis will be required to better characterize this trypanosomatid.

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### **BC62 - LEISHMANIA (VIANNIA) LAINSONI, AN ATYPICAL PARASITE OF THE VIANNIA SUBGENUS**

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INTRODUCTION - *Leishmania (V.) lainsoni* was described in Pará, Brazil

(Fernandes et al., 1987). It has been isolated from cutaneous lesions in humans and wild reservoirs, as well from the insect vector *Lutzomyia ubiquitalis*. This parasite presents hybrid morphological and biochemical characteristics from both the *Viannia* and *Leishmania* subgenera. Its biological behavior in axenic cultures and the way the intracellular amastigotes interact with the phagolysosomes of infected macrophages *in vivo* and *in vitro* do not coincide with the peripillary adhesion of the parasites in the insect vector, which a characteristic that has been used to include this protozoan in the *Viannia* subgenus. Aim of this work was to characterize the growth of *L. (V.) lainsoni* in axenic medium, as compared to *L. (L.) amazonensis* and *L. (V.) braziliensis*. Furthermore, we have analyzed by transmission electron microscopy the interaction between *L. lainsoni* and phagocytic cells *in vitro* and *in vivo*.

**MATERIALS AND METHODS** – Promastigote forms of *L. (V.) lainsoni*, *L. (L.) amazonensis* and *L. (V.) braziliensis* were grown at 25°C in NNN-LIT medium supplemented with 10% fetal calf serum. Cells were harvested daily and counted in Neubauer chamber, in order to establish the growth curves. *L. lainsoni* promastigotes were used to infect *in vitro* mouse peritoneal macrophages, as well as J774-G8 macrophages. After different times of interaction, the infected cells were collected, fixed with 2.5% glutaraldehyde in cacodylate buffer 0.1 M, post-fixed with 1% osmium tetroxide, dehydrated in graded acetone, embedded in epoxy resin, and observed by transmission electron microscopy (TEM). Chronic lesions were experimentally produced by the inoculation of *L. lainsoni* promastigotes in hamsters (*Mesocricetus auratus*). Lesion fragments were obtained and processed for TEM as described above.

**RESULTS** – The growth curve profile of *L. (V.) lainsoni* presents a distinct pattern for digenetic trypanosomatids, with promastigotes being produced in higher numbers during all the observation time (14 days), as compared to *L. (V.) braziliensis*, a typical *Viannia* member, and *L. (L.) amazonensis*, a typical *Leishmania* member. Observation by transmission electron microscopy allowed to demonstrate the close adhesion between the amastigotes and the phagolysosomal membranes, in both *in vitro* and *in vivo* infected macrophages. This morphological feature is typically observed in infections with amastigotes from the *Leishmania* subgenus.

**CONCLUSION** – Our results suggest that *L. (V.) lainsoni* presents biological characteristics from both the *Viannia* and the *Leishmania* subgenera. Furthermore, we demonstrate that axenic cultures of *L. (V.) lainsoni* can be easily maintained, and appear as an alternative to obtain in laboratory large cell masses for biological, biochemical and immunological assays. Further biochemical and molecular investigations are underway in order to better characterize the taxonomic position of *Leishmania (Viannia) lainsoni*.

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### **BC63 - PHYSIOPATHOLOGY OF BABESIA BOVIS: ADHESION MOLECULES EXPRESSED ON ENDOTHELIAL CELLS (ICAM-1, VCAM, PECAM-1, E-SELECTIN AND THROMBOSPONDIN)**

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Endothelial cells from bovine umbilical vein (BUVECs) were isolated with the purpose of determining the expression of ICAM-1, VCAM, PECAM-1, E-selectin and thrombospondin in the physiopathology changes of babesiosis caused by *B. bovis*. Later, this expression was confirmed in a *in situ* study, in tissue samples (brain, lung and kidney) of animals that died after inoculation with a pathogenic strain of *B. bovis* (BbovUFV1 7th passage). Erythrocytes of infected animals were tested in order to observer capacity of binding to BUVECs and its adhesion kinetics. The same adhesion tests were made on BUVECs

stimulated with plasma of animals infected with *B. bovis* and culture supernatant of bovine PBMC, stimulated with one synthetic peptide (SBb23290) derived from RAP-1 of *B. bovis* containing quantified cytokines (IFN- $\gamma$ , TNF- $\alpha$  and IL-12). There was a significant increase in the adhesion of erythrocytes of animals inoculated in BUVECs stimulated with plasma and supernatant of PBMC. However, adhesion was observed only on non-parasitised erythrocytes, suggesting that free antigens of *B. bovis* in the serum can prime erythrocytes non-parasitised, or still a possible expression of an isoform of VESA-1 non adherent. Adherence was not observed in the tests with samples of the negative animals. Cells stimulated with infected animals plasma and with supernatant of PBMC showed stronger expression of ICAM-1, VCAM, PECAM-1, E-selectine and thrombospondin, the cells that didn't receive stimuli not showed expression of adhesion molecules. In the same way, it was observed strong expression of ICAM-1, VCAM, PECAM-1, E-selectine and thrombospondin in tissue samples of brain, lung and kidney in bovines infected with *B. bovis*, when comparing to the control group. These results suggest that Interleukins, liberated in the acute phase of babesiosis, stimulate the expression of adhesion molecules related to the physiopathology of babesiosis caused by *B. bovis*, as demonstrated by the expression of molecules in BUVECs and erythrocytes cytoadhesion. These data demonstrate physiopathological similarities between *B. bovis* and *Plasmodium falciparum*.