RT1 – INVASION AND INTRACELLULAR BEHAVIOR IN PROTOZOA

RT1 - ALTERATION OF MACROPHAGE CELLULAR SIGNALING CASCADES BY *LEISHMANIA* PARASITES

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Leishmaniasis are worldwide spread syndromes affecting approximately 12 million people in at least 88 countries. Among several distinct clinical manifestations, diffuse cutaneous leishmaniasis (DCL) is of pivotal importance due to its frequent unresponsiveness to chemotherapy. Its pathology is characterized by non-ulcerated nodular lesions affecting most part of the body surface due to signaling defects in the skin immune system during early infection which promote a specific anergy and the exacerbation of the disease. It has been shown that serine¤threonine-asssociated signaling pathways are altered in infected macrophages which in turn fail to overcome infection (reviewed in Reiner 1994, Olivier 1986). Protein tyrosine kinase (PTK) activation in parasitic diseases has been largely overlooked despite its outstanding importance in phagocytosis (Greenberg 1995) and microbicidal processes (*e.g.* Dong et al. 1993). The infection of mononuclear phagocytes by *Leishmania* plays an important role in the pathophysiology of leishmaniasis, since this cell type not only sustains the parasite but also takes part in the immune response. Alterations in macrophage signaling may lead to defective immune regulation (Tapia et al. 1996). Several receptor-ligand systems have been implicated in the macrophage invasion by *Leishmania* sp. (Mosser & Rosenthal 1993), but little is known about the cellular responses triggered by the parasite binding and their relative role in its uptake and intracellular survival, as well as how parasites manage to subvert signaling cascades and thus impair effective immune responses.

Upon *L. amazonensis* binding to macrophage receptors an early and transient tyrosine phosphorylation of target proteins is triggered underneath the host cell plasma membrane. This response is restricted to contact areas between the promastigote flagellum and macrophage surface and participates in parasite uptake. Inhibition of phagocyte PTK activity by selective antagonists decreases both binding and uptake of parasites in a dose-dependent fashion (Martiny et al. 1996). The observations that virulent promastigotes induce a lower PTK activity than avirulent ones and that PTK inhibition diminishes NO release abrogating parasite killing strongly indicate that virulence factors might affect host cell PTK-mediated microbicidal mechanisms. Therefore this conceivable escape mechanism in leishmanial parasites was investigated in amastigote infection.

Internalization of amastigotes was inhibited neither by the tyrosine kinase antagonists nor by staurosporine, a broad spectrum protein kinase inhibitor and no immunolabeling using the 4G10 anti-phosphotyrosine monoclonal antibody could be observed at the parasite attachment areas, unless they were fixed with paraformaldehyde or pretreated with sodium orthovanadate. The reversion of tyrosine phosphorylation was assessed by Western blotting, in macrophages challenged with amastigotes for 60 min. Kinetic studies revealed that tyrosine dephosphorylation took place within 45 min and was completed by 120 min. After reversion of tyrosine phosphorylation in the first 2 hr it remains undetectable for at least seven days. Tyrosine dephosphorylation requires intact amastigote binding. Accordingly, no secreted phosphatase activity was detected during infection or incubation of parasites with macrophage-conditioned medium. Although not essential, internalization yielded a more efficient tyrosine dephosphorylation. In this regard it is interesting that uptaken parasites are found within the acidic mileu of the parasitophorous vacuole and that the ecto-phosphatase of amastigotes is optimally active at pH values ranging from 5.0 to 6.0.

The identity of target tyrosine dephosphorylated proteins was assessed by immunoprecipitation of infected and uninfected cells with agarose-conjugated 4G10 antibody. Five major phosphoproteins of 120, 85, 60, 44 and 35 kDa were detected in uninfected macrophages. All of them were mostly or completely absent in immunoprecipitates of infected cells. The treatment of amastigotes with sodium orthovanadate prior to infection restored the control pattern of phosphorylation and in some cases even led to a more intense phosphorylation. The overall tyrosine phosphorylation in infected cells was decreased by about 18-fold. Western blot analysis of immunoprecipitates revealed that p44 is the Erk1 mitogen-activated protein (MAP) kinase. The Erk1 MAP kinase was 120-fold enriched by vanadate pre-treatment of amastigotes. The reversion of Erk1 tyrosine phosphorylation by amastigotes could avoid its full activation, accounting for impairment of c-*fos* gene expression (Moore et al. 1993) and altered arachidonate synthesis (Reiner & Malemud 1985) previously described.

The existence of a parasite-derived phosphotyrosine phosphatase activity which could revert host cell activation was then examined. Intact amastigotes displayed a broad specificity, externally-oriented phosphatase activity largely sensitive to sodium orthovanadate (Martiny et al., manuscript in preparation) and about 10-fold higher than that of promastigotes (Vannier-Santos et al. 1995). It was recently described an ecto-protein tyrosine phosphatase activity in infective forms of *Trypanosoma cruzi* (Furuya et al. 1998) which is suggested to be linked to parasite virulence (Zhong et al. 1998). The amastigote ecto-phosphatase is currently being characterized by our group and it may also become a potential chemotherapic target. In this regard, vanadium derivatives have been successfuly tested in *in vitro* and *in vivo* models of murine leishmaniasis (Olivier et al. 1998). Taken together these results suggest that

Leishmania amastigotes actively revert PTK-linked signaling in macrophages resulting in a host inability to arrest leishmaniasis progression.

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RT1 - MAMMALIAN CELL INVASION BY *TRYPANOSOMA CRUZI* AND SIGNAL TRANSDUCTION

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The virulence of many microorganisms pathogenic to humans is associated with their ability to invade host cells. Recent studies have revealed that internalization of these pathogens requires activation of host cell signal transduction pathways. Thus, events such as phosphorylation of target cell proteins and Ca^{2+} mobilization, triggered by binding of microbial ligands, have been demonstrated to play an important role in the process of cell invasion. In the case of the protozoan parasite *Trypanosoma cruzi*, the etiological agent of Chagas' disease, protein tyrosine kinase activity of both the parasite and the host cell is implicated in *T. cruzi* endocytosis by macrophages (Vieira et al. 1994). Also required for *T. cruzi* entry into various mammalian cell types is the target cell as well as the parasite Ca^{2+} mobilization (Moreno et al. 1994, Tardieux et al. 1994, Yakubu et al. 1994, Dorta et al. 1995, Barr et al. 1996, Wilkowsky et al. 1996).

We have observed that the ability to enter mammalian cells may differ significantly in metacyclic trypomastigotes of different *T. cruzi* strains (Ramirez et al. 1993). To determine whether such a variability reflected differential expression in *T. cruzi* strains of surface molecules with differential Ca^{2+} signaling activity, we examined in metacyclic forms of different strains: (i) the expression of surface glycoproteins, in particular of gp35/50, gp82 and gp90, previously known to interact with host cells (Yoshida et al. 1990, Ramirez et al. 1993, Ruiz et al. 1993) and (ii) the ability of these molecules to induce Ca^{2+} signal in parasites and host cells.

Mammalian cell invasion assays, using metacyclic forms of *T. cruzi* G and CL strains, showed that CL strain enters target cells in several fold higher numbers as compared to the G strain. Analysis of expression of surface glycoproteins in these strains revealed that: (i) gp90, undetectable in CL strain, is one of the major surface molecules in G strain, (ii) expression of gp82 is comparable in both strains, and (iii) gp35/50 is expressed at lower levels in CL strain. Purified gp90 and gp35/50 bound more efficiently than gp82 to cultured HeLa cells. However, the intensity of Ca^{2+} response triggered in HeLa cells by gp82 was significantly higher than that induced by gp35/50 or gp90. Ca^{2+} mobilization was also triggered in metacyclic trypomastigotes by host cell components, it was mainly gp82-mediated and more intense in CL than in G strain. Analysis of metacyclic forms of 8 additional *T. cruzi* strains corroborated the inverse correlation between infectivity and expression of gp90 and gp35/50 (Ruiz et

al. 1998). We propose that expression of gp90 and gp35/50 at high levels impairs binding of metacyclic forms to host cells through gp82, a metacyclic stage-specific surface glycoprotein implicated in target cell invasion (Ramirez et al. 1993) which mediates target cell and parasite Ca^{2+} mobilization required for invasion.

In an attempt to determine the events upstream to Ca^{2+} mobilization, we examined the requirement of *T. cruzi* protein tyrosine phosphorylation for parasite entry into mammalian cells and analysed the profile of phosphorylated proteins in infective trypomastigotes. Treatment of metacyclic or tissue culture trypomastigotes with 250 mM genistein, an inhibitor of protein tyrosine kinase activity, significantly inhibited invasion of cultured HeLa cells. A soluble factor, contained in HeLa cell extract and absent in the extract ot *T. cruzi*-resistant K562 cells, greatly enhanced phosphorylation levels of a 175 kDa protein (p175) in trypomastigotes. Genistein inhibited p175 tyrosine phosphorylation. P175 was undetectable in non invasive epimastigotes. The phosphorylation-inducing activity of HeLa cell extract was abrogated by adsorption with metacyclic trypomastigotes but not with epimastigotes, or when it was mixed with recombinant protein J18, which contains the entire peptide sequence of gp82. These data suggest that, in metacyclic trypomastigotes, gp82 is the signaling receptor that mediates protein tyrosine phosphorylation necessary for host cell invasion.

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RT1 - THE FLAGELLAR POCKET OF TRYPANOSOMATIDS (OR, HOW TO EAT WITH YOUR MOUTH CLOSED)

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The majority of protists exhibit a level of intracellular compartmentalization that is more obvious than that of individual mammalian cells. This intracellular specialization has developed from the need to fulfill diverse functions, carried out by organs in a multi-celled animal, within a single cell. Amongst the trypanosomatids, this need to compartmentalize is augmented by constraints dictated by a parasitic existence. One obvious problem to be overcome is the acquisition of nutrients through the cell surface without exposure to elements of the host's humoral (*Trypanosoma brucei*) or lysosomal (*Leishmania* spp.) systems. Several transporter systems for uptake of sugars, amino acids and ions have been demonstrated on trypanosomatid surfaces, but it is the need to internalize macromolecules that could lead to exposure of the parasite to host defences. In most trypanosomatids the cytostome and flagellar pocket have combined to form a protected compartment that apparently facilitates access to nutrients, while extending some degree of protection to the receptors required for uptake of these molecules. In addition to endocytosis, this region of the cell is also the site of secretion and addition of plasmalemma constituents to the cell membrane.

Although there are undoubtedly differences between the function of the flagellar pocket in different trypanosomatids many "housekeeping" properties will be conserved. We have been examining both Leishmania and *T. brucei* in parallel to allow conserved versus parasite-specific molecules to be identified.

Results and Discussion

We have developed methods for isolating cellular fractions highly enriched for the endosomal compartments of trypanosomatids. These fractions were used to generate panels of monoclonal antibodies against antigens that, by immunofluorescence and immunoelectron microscopy, show a distribution consistant with a role in regulation of pocket funmction or in endocytosis/endosome maturation. We have currently cloned and sequenced genes encoding two of these antigens and are targeting these two proteins for further functional characterization.

FLAP antigen. Antibody 12F9 was used to identify and isolate three clones from a *L. mexicana* amastigote cDNA library. The clones all contained overlapping inserts of up to 2.5 kbp. Sequencing of these clones revealed a novel gene which showed homology to the mammalian cell/cell junction protein desmoplakin. This homology

was observed in regions predicted to possess alpha coil/coil structure, common to many cytoskeletal, rod-like proteins such as myosin, as well as other regions of the sequence. The protein's abundance and it's structure suggests that it fulfills a major structural role in the formation of the junction complex and may act as na anchor for other elements of the junction.

The cDNA clones were used to probe southerns and genomic libraries. The southern pattern indicated a single gene copy in *Leishmania, T. brucei* and *Crithidia*. We now have genomic clones that span the entire gene, and have sequenced around 85% of the gene, although we have had difficulty sequencing through an extended region of degenerate repeats located near the 5' end of the gene. Antibody raised against the recombinant protein gives the same pattern as 12F9 by immunoblot and immunofluorescence confirming the identity of the gene. The prediction, given the gene structure and the localization of the protein to the mouth of the flagellar pocket, is that this protein represents a major constituent of the hemidesmosomal junction. This is the only protein identified in this structure to date and the cloning of its gene opens the way to a unique approach to analysis of the flagellar pocket function in these trypanosomatids.

I278 antigen - This protein localizes to the endosomes and multivesicular lysosomes of bloodstream of *T. brucei*. N-terminal sequence from the intact and digested protein were used to design primers and we were able to PCR amplify independently two fragments from genomic DNA, and have subsequently cloned the gene which encodes a type two membrane protein predicted to have a 21 amino acid cytoplasmic tail. The sequence is unrelated to any trypanosomatid sequence in the database or in the EST libraries.

Given the localization of the antigen to the limiting membrane of both endosomes and lysosomes we are particulary interest in gaining information with respect to the cytosolic tail/domains of the protein. This information has tremendous potential for both appreciation of the protein sorting mechanisms (ie adaptin-binding motifs) and as use for generating probes for the affinity-isolation of these compartments.

Conclusion

This lecture will discuss the position of our ongoing studies on these two proteins with respect to current appreciation of the fole of the flagellar pocket and the endocytic network in protein secretion andthe acquisition of nutrients by both *Leishmania* and *T. brucei.*

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RT1 - HOST CELL INVASION BY *TOXOPLASMA GONDII*

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Invasion of the host cell by *Toxoplasma gondii* leads to the internalization of the parasite into a membrane bounded parasitophorous vacuole where the parasite multiplies by a complex binary fission process called endodyogeny. A major property of the vacuole is that it never fuses with any other compartment of the host cell [1]. This property is explained by the origin of the vacuole: *T. gondii* invasion is an active process depending on the actin based gliding motility of the parasite and does not depend on host cell activity such as phagocytosis [2]. The parasite is likely to uses the host cell plasmalemma to create the vacuole [3] but modifies it in such a way that it can no longer interact with the endocytic system of the host cell. How this works is not fully understood. What has been shown is that the host cell plasmalemma is continuous withthe developing vacuole membrane during all the invasion process, through a zone of close contact between the two plasmalemmas, corresponding to a structure named moving junction. The early vacuole membrane is devoid of intra membranous particles, suggesting that host cell proteins do not cross the moving junction. Host cell surface iodination or biotinylation followed by invasion and then analyzed by high resolution autoradiography or cryoimmunoEM further show that surface molecules of the host cell are not internalized into the vacuole membrane.

As all the invasive stages of Apicomplexa, tachyzoites possess 3 types of apical organelles (micronemes, rhoptries, dense granules) that are synthesized during endodyogeny. The biosynthetic pathways of these organelles are actively investigated. These pathways are sensitive to Brefeldin A, and two of them (rhoptry and micronemes) undergo proteolytic processing events in intermediate biosynthetic compartments [4,5]. These secretory organelles are sequentially exocytosed during the invasion process [6].Micronemes are released during the early steps and are likely to be involved in gliding and binding to substrate and host cell. Three microneme proteins have been identified so far, that possess homologies to higher eukaryote adhesins [7,8] and may be responsible for host cell recog-

nition and adhesion. Rhoptry proteins are released during the internalization and become associated with the developing vacuole membrane, some as peripheral, others as transmembrane.Their functions are not known. Phospholipase activities have been suggested to be involved in invasion and may originate from the rhoptry contents [9]. Some rhoptry proteins may also be involved in the association of host cell mitochondria with the vacuole membrane [10] or with the formation of transmembrane pores that ensure parasite-host cell metabolic exchange in the absence of vesicular fusion [11].The dense granules contents are exocytosed in the newly formed vacuole and during all the intracellular development and they associate with the vacuole membrane or with an intravacuolar tubular network that forms after invasion [12]. Dense granule proteins functions are not known apart for one NTPase that is likely to be involved in host cell nucleotide cleavage to allow nucleoside uptake by the parasite [13,14]. The complex series of events occuring sequentially during the invasion process must involve a complex pattern of signalization, that is yet entirely unknown.

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RT2 – NEW APPRACHES FOR DRUG DESIGN

RT2 - NEW INHIBITORS OF THE GLYCOLYTIC PATHWAY AS POTENTIAL ANTICHAGASIC DRUGS: VALIDATING THE TARGETS FOR ANTIPARASITIC CHEMOTHERAPY

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Modern drug discovery involves a multidisciplinary approach including the areas of medical and biological sciences, genomics, chemistry, biomolecular structure, computational chemistry, pharmacology and clinical trials. A broad project as such is being developed by an interinstitutional team, having as target the glycosomal enzyme glyceraldehyde-3-phosphate dehydrogenase from *Trypanosoma cruzi*. The final goal is to find molecules with potential anti-chagasic activity. The enzyme was cloned and overexpressed in *Escherichia coli*, purified, crystallized both on Earth and under microgravity conditions, and its structure was determined to high resolution. The techniques of structure based drug design were applied, focusing at the NAD cofactor binding site. Several of the designed compounds were synthesized and have shown significant inhibiton of the enzyme and *in vitro* trypanocidal activity. We are also conducting a broad screening of natural products from the brazilian biodiversity, in search of lead compounds for further development. This innovative approach combines the identification and purification of compounds from crude extracts, guided by the enzymatic inhibitory assays and *in vitro* testing, and also the systematic screening of previously purified natural products. Several substances with excelent activity were so far found and are now the focus of optimization by rational design. Complementarly, site-directed mutants were planned based on the crystal structure, prepared and produced by recombinant techniques, analysed with respect to their enzyme kinetics, with the objective of better understanding the catalytic mechanism of the target enzyme and to identify alternative target inhibition sites at the molecular surface.

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RT2 – PROTEIN PRENYLATION IN TRYPANOSOMATIDS: DEVELOPMENT OF ANTI-PARASITIC DRUGS USING A "PIGGY-BACK" MEDICINAL CHEMISTRY APPROACH

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The enzymes and receptors in parasites that can be qualified as targets for antiparasitic chemotherapy should perform essential functions in the parasites and demonstrate a feasibility for selective inhibition. They can be identified through detailed metabolic analysis of the parasite or by elucidation of the mechanisms of action among proven antiparasitic agents. Preliminary verification of these putative targets can be indicated by the in vitro antiparasite activity of the inhibitors of the targets. However, an individual chemical compound often exerts multiple effects on a variety of receptors and enzymes. Before a major commitment to an in-depth structure-function analysis of a target for specific inhibitor design, further validation of the target will be essential. One old-fashioned approach to validate a target in the pharmaceutical industry is by correlating target inhibitions with antiparasitic activities among a large number of inhibitor derivatives. The results are often indicative but hardly ever conclusive. Another method is by comparing the putative drug targets between the drug-sensitive and the drug-resistant parasites for potential discrepancy. Unfortunately, the latter often result from indirect causes, such as reduced drug transport, instead an alteration of the target itself. The third experimental approach is by disrupting the gene encoding the putative target in parasite, which should provide the most convincing evidence on whether the target plays an indispensable role in the parasite. But special experimental conditions are needed for gene-knockout mutants to survive to exhibit specific phenotypes and to allow genetic complementation for further verification.

Among the members of Kinetoplastidae, one of the chemotherapeutic targets that has been best defined and fully validated by the most strict criteria is the ornithine decarboxylase in *Trypanosoma brucei*. By isolating the gene-knockout mutants of *T. brucei* and performing genetic complementation studies, conclusive evidence has been obtained which demonstrates that the function of ornithine decarboxylase is essential for the growth of *T. brucei*. Ironically, this indispensable enzyme function can be only attributed to the particular living environment of African trypanosomes, where there is little exogenous polyamines available for import. Thus, for the other members of Kinetoplastidae which inhabit in an polyamine-enriched environment, their ornithine decarboxylases cannot be validated as targets for chemotherapy. There is also little obvious opportunity for designing specific inhibitors against *T. brucei* ornithine decarboxylase due to the 62% sequence identity between the human and *T. brucei* enzymes. But the *in vivo T. brucei* ornithine decarboxylase activity is unusually susceptible to inhibition by ubiquitous suicide inhibitors of ornithine decarboxylases, such as difluoromethylornithine, because it has a very long half-life comparing with that of the mammalian ornithine decarboxylase. The enzyme-structure-based specific inhibitor design is thus unnecessary in this particular case. The unique machinery of protein degradation in *T. brucei* has apparently validated its ornithine decarboxylase as an easy target for inhibition *in vivo*.

RT2 - ANTI-TRYPANOSOMATID TARGETS: GLYCOLYSIS AND PROTEIN PRENYLATION

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We have shown that protein prenylation (farnesylation and geranylgeranylation) occurs in trypanosomatids, and we have purified to homogeneity the protein farnesyltransferase from *Trypanosoma brucei*. Inhibitors of this enzyme block protein farnesylation in trypanosomatids and sub-micromolar concentrations are lethal to cultured *T. brucei* , *T. cruzi*, and *L. mexicana*. Since there is an enormous effort to develop protein farnesyltransferase inhibitors as anti-cancer agents, one may be able to extend the medicinal chemical and clinical pharmacological studies currently in progress to the development of anti- trypanosomatid agents.

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RT2 - EXPLOITING *TRYPANOSOMA CRUZI* AS A SOURCE OF NATURAL PROTEIN INHIBITORS OF CYSTEINE-PROTEINASES: THE CASE OF CHAGASIN

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Genes encoding for papain-related cysteine proteases (CP's) have been identified in several species of parasitic protozoa and helminths, and important functions have been attributed to them. Instead of being viewed as strict digestive proteinases, the papain-like CP's expressed by parasites seem to have evolved to perform multiple tasks, in different hosts, and environmental settings. In parasitic protozoa, the vast majority of well-characterized CP's are confined to vacuolar or lysossomal-like organelles Apart from the general rubrique of protein catabolism, little is known about their precise biological roles *in vivo*. In the *Trypanosoma cruzi* species, a large number of closely related genes encode for cathepsin L-type of CP's, collectively designated as cruzipain (cruzain). Although the expression of cruzipain genes is regulated by post-transcriptional mechanisms, the molecular basis of these events is still obscure. Seeking for clues, we sought to identify cysteine proteinase inhibitors from *T. cruzi*. This was accomplished by screening of epimastigote (Dm28c) lgt11 cDNA expression library, using ligand binding to carboxy-methylated papain and cruzipain. Several full-length clones encoding the inhibitor, named chagasin, were identified and the 5' non-coding sequence up to the *T. cruzi* miniexon sequence was determined by reverse transcriptase PCR of epimastigote mRNA. The amino acid sequence of chagasin is composed of 110 amino acids (Mr 12,039), lacks cysteine residues and is devoid of the QXVXG motif characteristic for cysteine proteinase inhibitors belonging to the cystatin superfamily. Northen blotting revealed differential expression of chagasin mRNA during *T. cruzi* development (trypo>amas>epi). A 126-residue recombinant form of chagasin (r-chagasin) was produced in high yields in a periplasmic *Escherichia coli* expression system and isolated. The far-UV circular dichroism spectrum of purified r-chagasin was clearly different from that of cystatin C, and secondary structure predictions indicated a much lower proportion of ordered structure for chagasin than for family 2 cystatins. By analogy to cystatins, however, the *T. cruzi* inhibitor formed 1:1 reversible complexes with papain, cruzain (UCSF), cathepsin B, and cathepsin L. The interactions resulted in high-affinity binding of all enzymes (Ki values of 0.023, 0.0049, 1.9, and 0.018 nM, respectively). Recombinant chagasin also displayed potent inhibitory when tested with r-cruzipain 2, an isoform which is only weakly inactivated by cystatins and E-64 (Lima et al. 1998, Dos Reis et al. 1998 this issue). A monospecific antiserum against r-chagasin was then used to identify natural chagasin (n-chagasin) in *T. cruzi* lysates. Consistent with Northern blotting analysis, the immunoreactive protein (~11KDa) was differentially expressed during parasite development, being present in reduced quantities in epimastigotes. We then used affinity chromatography to isolate n-chagasin from R-Dm28, a *T. cruzi* cell line that produces fairly low amounts of endogenous cruzipain (Yong et al. 1998, this issue). The purified protein bound reversibly to cruzipain and displayed a Ki value (Ki 0.0024 nM) similar to r-chagasin. Overlapping peptides spanning the full-length primary sequence of chagasin were then synthesized and screened for inhibitory activity. Our data suggest that 2 distinct linear segments of this protein may be possibly involved in the inhibitory mechanism. We may antecipate that the knowledge stemming from analysis of chagasin structure might offer new clues for the design of synthetic inhibitors to papain-like proteinases, perhaps not limited to the field of Chagas' disease. For example, we have recently observed that r-chagasin can drastically impair *Schistosoma mansoni* mansoni metabolism due to efficient inactivation of their digestive gut's hemoglobinases. Additional applications of this research will be discussed.

Supported by MCT (PRONEX II).

RT3 - IMMUNOPATOLOGY IN CHAGAS' DISEASE

RT3 - THE SUPPRESSOR FUNCTION OF gd T CELLS DURING THE ACUTE PHASE OF *TRYPANOSOMA CRUZI* INFECTION MIGHT PREVENT THE DEVELOPMENT OF AUTOIMMUNITY IN THE CHRONIC PHASE

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A growing body of evidence from different experimental models confirms and extends the notion that gd T cells may may be involved in the induction and maintenance of tolerance. For instance, it has been reported that ab+ CD4+ T cells from gd T cell-depleted mice have increased levels of IFN-g and IL-2 production and thymidine incorporation when cultured with syngeneic filler cells (1, 2). Hepatic gd T cells from mice made tolerant by the transfer of spleen cells from minor histocompatibility-mismatched mice have been demonstrated to confer tolerance to naive recipients (3-5). gd T cell-depleted mice are not susceptible to the induction of oral tolerance to ovalbumin, and that once established, oral tolerance could be terminated by depleting gd T cells (6).

Recently, we showed that splenic gd T cells from young BALB/c mice possessed a suppressor activity in vivo and *in vitro* during the acute phase of *Trypanosoma cruzi* infection (7). The gd T cell suppressor activity found in these animals was not dependent on the presence of IL-4, IL-10 or TGF-b in its effector phase. Moreover, it was demonstrated that the spontaneous release of interferon-g by ab T cells from anti-gd mAb-treated infected mice was greatly enhanced when compared with non-treated infected mice. In clear contrast to this, non-adherent spleen cells from aged BALB/c mice infected with *T. cruzi* did not show any suppressor activity, in spite of the fact that comparable percentages of the different lineages and phenotypes of T cells was found in the spleen of both young and aged *T. cruzi* infected

mice. The lack of gd T cell suppressor activity in aged *T. cruzi*-infected mice was correlated with a more severe myocarditis in the chronic phase of the infection, thus suggesting that this suppressor mechanism could control the development of the anti-self myocardial immune response found in the chronic phase of *T. cruzi* infection (8).

The ageing process is accompanied by thymic involution which results in a considerable decrease in the number of emigrant thymic cells (9). Based on the observation that the gd T cells from aged *T. cruzi*-infected mice do not present suppressor activity and that aged mice are thymic deficient, we designed experiments to study the influence of the thymus on the suppressor activity of gd T cells from *T. cruzi*-infected mice. In relation to this, we show that splenic gd T cells from athymic BALB/c nude mice, reconstituted or not with peripheral ab T cells do not present suppressor activity, *in vitro*, during the acute phase of *T. cruzi* infection. Moreover, thymectomy of young BALB/c mice induced a progressive, time-related reduction in the suppressor activity of nonadherent spleen cells from *T. cruzi*-infected young BALB/c mice. On the other hand, the continuous provision of thymocytes to aged mice, young thymectomized mice or T cell-reconstituted athymic nude mice could reestablish the gd T cell suppressor activity. Further evidences that the suppressive activity of gd T cells is operating *in vivo* come from the fact that *T. cruzi*-infected mice could not mount a primary humoral immune response to a non-related antigen such as ovalbumin. However, the depletion of gd T cells restored the ability of infected mice to make an anti-ovalbumin specific humoral primary immune response comparable to non-infected controls. This latter finding suggests that suppressor gd T cells are not specific for the nominal antigen, but are able to suppress any non-related immune response that may appear during this period. This mechanism could, theoretically, control clonal activity of other peripheral ab T cells, thus down regulating parasite-specific or parallel imune responses to unrelated or even self antigens

The suppressor activity of gd T cells is likely to participate in the control of the immune response to *T. cruzi* antigens, since the absence of suppressor gd T cells resulted in a more effective host immune response to the parasite, as indicated by the relative resistance (lower levels of parasitemia and an increase in survival) of aged or thymectomized mice along the acute infection.

Taken together, the results of the present study reinforce the notion that gd T cells may have or acquire different effector functions, depending on the origin (thymic or peripheral) or as yet unknown interactions with other cells. Our results also indicate that gd T cells of extrathymic origin can not mediate suppression in the absence of a functional thymus and that the thymus has a role in the regulation of suppression as well as in the determination of resistance or susceptibility, during the acute phase of *T. cruzi* infection. Furthermore, this suppressor mechanism could control the development of the anti-self myocardial immune response found in the chronic phase of *T. cruzi* infection (7,8, 10).

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RT3 - AUTOREACTIVE CD4+ T CELLS IN THE PATHOGENESIS OF THE CHRONIC MYOCARDITIS FOUND IN THE EXPERIMENTAL *TRYPANOSOMA CRUZI* INFECTION

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Chagas disease, caused by the protozoa parasite *Trypanosoma cruzi*, affects up to 18 million people, being one of the leading causes of death in many countries of Latin America. The most striking feature of the disease is the heart inflammation during the chronic phase, predominantly composed by mononuclear cells and intersticial fibrosis with rare tissular parasitism. Several studies suggest that autoimmune mechanisms play a role in the fisiopathology of the chronic myocardites in Chagas disease. We have previously shown, in a heart transplant model (newborn heart were grafted in the pine of ear of syngeneic mice), that mice chronically infected with the Colombian strain of *T. cruzi* consistently reject the transplants. In addition, strong evidences indicated that CD4+ T cells were responsible for such a phenomenon: (i) the rejection was dependent on CD4+ T cells since anti-CD4 but not anti-CD8 mAb treatment abrogated rejection and CD4+ but not CD8+ T cells from chronically infected mice induced rejection when injected into well-established transplants. (ii) Purified splenic CD4+ T cells from chronically infected mice

proliferate to myocardium antigens *in vitro*. (iii) *In vivo* treatment of chronically infected mice with anti-CD4 mAb could prevent newborn graft rejection, thus re-establishing self tolerance to myocardium antigens either *in vivo* or *in vitro*.

Contrary to our results, it has been recently demonstrated long term engraftment of syngeneic heart transplants into mice chronically infected with the Sylvio strain of *T. cruzi*. In this study, parasitism of heart tissues was necessary and sufficient for the induction of inflammatory response, tissue damage and graft rejection. The present study was designed to better characterize CD4 T cell autoreactivity as the cause of rejection of newborn heart tissues transplanted into mice chronically infected with *T. cruzi* of the Colombian strain. Our results demonstrated that CD4+ T cells predominate in heart inflammatory infiltrate during the chronic phase of *T. cruzi* infection. The percentage of CD4+ or CD8+ T cells in the heart inflammatory infiltrate was evaluated by FACS analysis. Inflammatory heart cells were stained with anti-CD4 or anti-CD8. The results show that during the chronic phase of *T. cruzi* infection (eight months post-infection) the percentage of CD4+ T cells (36% among gated lymphocytes) was higher than the percentage of CD8+ T lymphocytes (25.2% among gated lymphocytes). Furthermore, a CD4+ T cell line (RRSJM-1) was generated by repeated in vitro antigenic stimulation of purified splenic CD4+ T lymphocytes from chronically *T. cruzi*-infected mice. RRSJM-1 cells proliferate in the presence of soluble heart antigens and syngeneic feeder cells or in co-cultures with irradiated splenic syngeneic feeder cells and fetal heart cells. The cell line could also decrease the number of beating fetal heart cell-clusters *in vitro* when co-cultured with irradiated splenic syngeneic feeder cells and fetal heart cells. In addition, in situ injection of the RRSJM-1 cells into well established heart transplants induces cessation of heart beating. PCR analysis of RRSJM-1 cells and of the rejected heart transplants excised 10 days after the injection of RRSJM-1 cells reveal no *T. cruzi* positive amplified product.

To further study the role of autoreactivity in the pathogenesis of the myocardium heart plesions, RRSJM-1cells or normal splenic CD4+ T cells were transferred to BALB/c nude mice. Histological studies of their hearts, two months after the cell transference, revealed the presence of multifocal mononuclear infiltrates, similar to those observed during the chronic phase of *T. cruzi* infection, although less intense. No significant alterations were observed in the hearts of BALB/c nude mice transferred with normal splenic CD4+ T cells. Mice transferred with RRSJM-1 cells did not show any signs of infection as judge by PCR or parasitemia.

In conclusion, the findings in the present study allow us to suggest that autoimmunity is the major mechanism implicated in the rejection of syngeneic heart tissues grafted into the pinna of the ear of mice chronically infected with the Colombian strain of *T. cruzi*.

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RT3 - CHECKPOINTS FOR AUTOIMMUNITY-INDUCED HEART TISSUE DAMAGE IN HUMAN CHAGAS' DISEASE: A MULTISTEP PROCESS?

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The pathogenesis of CCC is under intense debate (1,2). Even though heart-infiltrating T cells have been implicated as the ultimate effectors of heart tissue damage, a direct role for heart parasitism has been proposed after the identification of *Trypanosoma cruzi* antigen and DNA in CCC hearts by immunohistochemical and PCR techniques (3,4). However, low-grade parasitism is widespread in several other organs like the kidneys (5,6) as well as among hearts from "asymptomatic indeterminate" (ASY) individuals (7) which nevertheless do not suffer from any major functional damage. The major histopathological feature attending dilated cardiomyopathy in CCC is the presence of a diffuse myocarditis, with intense tissue damage (8) and scarce *T. cruzi* forms. On the other hand, a focal myocarditis can be observed in a significant number of samples from ASY patients (8) often in association with *T. cruzi* antigen (2). This might indicate that while direct *T. cruzi* tissue parasitism might induce some inflammation, the presence of *T. cruzi* in tissue may not be necessary and sufficient for heart damage. Among CCC, but not ASY, patients, the heart infiltrate seems to undergo a transition to pathogenic potential leading to dilated cardiomyopathy, in a process similar to that expressed in models of autoimmune disease (9,10). Susceptibility factors leading 30% of infected patients to undergo such transition are largely unknown, as the mechanisms of the transition are still obscure.

T cells infiltrating the heart of CCC patients possess an inflammatory T_1-type cytokine profile (11) and some T cell clones obtained from *T. cruzi*-free heart biopsies crossreactively recognize cardiac myosin and *T. cruzi* antigen B13 (12), suggesting that heart damage was secondary to inflammatory cytokines and a delayed-type hypersensitivity process started or maintained by heart-crossreactive T cells. The cytokine balance measured via the response of PBMC from CCC or ASY individuals to B13 protein disclosed high levels of IFN-g and negligible IL-4 production (13). This systemic shift to a T_1-type cytokine profile is probably related to the IL-12 inducing activity of trypomastigote mucin glycoconjugates along chronic *T. cruzi* infection (14). However, this polarized T_1-type of cytokine response is not sufficient for heart tissue damage since ASY individuals share this feature but do not display diffuse myocarditis with tissue damage.

We recently identified that PBMC from normal individuals display MHC-restricted proliferative responses to *T. cruzi* B13 protein similar in frequency and intensity to those found among *T. cruzi* infected individuals. Preliminary database searches with B13 epitope motifs identified several homologous food or pathogen antigenic epitopes, suggesting that normal individuals may be sensitized to different environmental or self-antigens (possibly distinct from cardiac myosin) in molecular mimicry with *T. cruzi* B13 protein. Cardiac myosin-recognizing T cell clones could be recovered from T cell lines induced with B13 from peripheral blood of a normal individual (15), implicating that i. sensitization with B13 *in vitro*, or perhaps *in vivo* along *T. cruzi* infection, may prime T cell clones able to recognize cardiac myosin; ii. myosin-B13 crossreactive clones do not carry pathogenic potential in normal individuals. Studying B13-induced cytokine responses from PBMC of normal individuals, it was found that roughly 40% produce IL-4 while other 40% produce low levels of IFN-g (Abel LCJ et al. submitted), indicating an important heterogeneity among individuals prior to *T. cruzi* infection which could potentially affect the immediate handling of parasitism (16,17).

Thus, it can be hypothesized that the generation of a pathogenic T cell infiltrate in Chagas disease cardiomyopathy is a multistep process where pre-infection immunological features of the host, like the cytokine balance (16,17) or the crossreactive T cell recognition repertoire (15,18), are major determinants. Systemic, rather than local, chronic *T. cruzi* infection may function as a trigger and booster, generating "experienced" pathogenic T cells in susceptible individuals that may subsequently migrate to the heart and induce tissue damage.

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RT4 – ADVANCES IN MOLECULAR AND POPULATION STUDIES ON VECTORS

RT4 - CURRENT STATUS OF THE *LUTZOMYIA LONGIPALPIS* SPECIES COMPLEX

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While it is generally accepted that *Lutzomyia longipalpis* (Lutz & Neiva 1912) is a complex of morphologically indistinguishable species, the number involved is unknown and several studies have sought to resolve this problem using different methods. Conflicting evidence based on two such techniques (isozyme analysis and sex pheromone analysis based on gas chromatography) is presented and compared in this review.

The geographical distribution of *L. longipalpis sensu lato* extends from central Mexico to northern Argentina and Paraguay[1]. It is apparently absent from the Pacific Coast of South America, there being no records from Ecuador, Peru or Chile. This suggests that the Andes acted as a barrier to the spread of this sand fly, although it was able to penetrate the length of the Magdalena valley in Colombia, which divides the Eastern and Central Cordilleras[2]. Although most New World sand flies are associated with forested areas, *L. longipalpis* has adapted to disturbed, treeless and even peri-urban habitats, where it is often associated with domestic animal shelters. The public health significance of this sand fly lies in its role as the vector of *Leishmania chagasi*, causative organism of visceral leishmaniasis. This relationship occurs throughout the wide geographical distribution of *L. longipalpis*, with the possible exception of Panama. The single report of a VL case from Ecuador is doubtful, since *L. longipalpis* appears to be absent from that country[3]. Both vector and parasite are present in Costa Rica but here a nodular cutaneous form of the disease is manifest in humans, possibly linked to the absence or reduced activity in Costa Rican sand

flies of maxadilan, a salivary peptide that is a potent vasodilator and may aid in visceralization of *Le. chagasi*[4].

The first report of morphological differences between populations of *L. longipalpis* was by Mangabeira[5] who noted differences in the number of pale patches on the male abdominal tergites. Ward et al[6] carried out cross-mating experiments using laboratory-reared flies from different parts of Brazil bearing one or two pairs of these tergal patches, the results of which indicated *L. longipalpis* to be a complex of at least two species. The spots were later found to overly pheromone-producing glands[7] and two sex pheromones (a diterpene and a farnesene/homofarsene) were described from members of the complex[8]. Subsequent research revealed that pheromone type produced was not dependent on the number of tergal spots, all four possible combinations of pheromone type and spot number being present in nature[9].

Lanzaro et al.[10] analyzed genetic variability in laboratory colonies of *L. longipalpis* from Costa Rica, Colombia and Brazil at 27 enzyme coding loci and found high values for the genetic distances (D) among these populations. Cross-mating experiments resulted in the production of sexually sterile male progeny, providing additional evidence that each population represented a distinct species. Further studies[2] using 16 enzyme loci revealed low genetic distances (D = 0.021) among wild-caught populations from three localities (up to 600 km apart) in the Colombian Andes but confirmed the differences between Colombian populations of *L. longipalpis* in general and those from Costa Rica and Brazil (D = 0.199 and 0.098 respectively). Genetic distances between the Colombian field populations and the laboratory population (Melgar, Department of Tolima) used in the Lanzaro et al.[10] study were low (D = 0.016), confirming that individuals in this long-established colony were not significantly different from wild insects.

Studies on *L. longipalpis* from 11 Brazilian localities, again using 16 enzyme loci, revealed that gene frequencies within populations conformed closely to Hardy-Weinberg expectations, indicating no sympatric species. Levels of genetic distance between pairs of populations were very low (< 0.03) consistent with the view that all Brazilian *L. longipalpis* belong to one species. When genotypic data from all populations were pooled, 9 of the 13 polymorphic enzyme loci deviated from Hardy-Weinberg expectations, indicating some degree of genetic substructuring. This is probably explained by the low effective migration rate (Nm = 2.6), which showed gene flow among populations to be resticted.

Results of isozyme analyses therefore conflict with those obtained through chemical analysis of male sex pheromones in defining the number of species in the *L. longipalpis* complex and their respective distributions. Recent studies[11] have indicated that there are at least three different types of sex pheromone. These compounds are a novel series of terpenes, characterized as the methylsesquiterpenes (C16) 9-methylgermacrene-B and 3-methyl-a-himalchalene and the monocyclic diterpene cembrene or possibly cembrene-B (C20). The results of laboratory experiments indicate that females are only attracted to the pheromone type produced by conspecific males.

From re-examination of Ward et al.'s[6] cross-mating experiments it appears that successful or unsuccessful crosses can be explained by the effect of three different sex pheromones. Interestingly, where forced cross-matings take place between insects of different chemotypes, the resultant F1 males produce both sex pheromones in approximately equal amounts. No hybrid males of this type were found among wild-caught specimens from areas where the two chemotypes were sympatric.

In addition to the three Brazilian sibling species based on semiochemical evidence, a fourth member of the complex may occur in Costa Rica, where an additional novel methylsesquiterpene has been found in male *L. longipalpis*. Laboratory cross-attraction studies using insects from two 3-methyl-a-himalchene-producing populations suggest that the quantity of pheromone produced by males may also play a role in species isolation. Males from Lapinha (MG) and Sobral (Ceará) both produce 9-methylgermacrene-B but the former are significantly larger and produce more pheromone than the latter. Similar differences have been seen between cembrene-producing males from Marajó and Santarem (Pará).

However many species occur within the *L. longipalpis* complex, it is not clear whether the present-day distribution of these insects is expanding, as they colonize deforested areas and shanty towns surrounding neotropical cities, or whether it represents a remnant of the area formerly occupied, as suggested in Adler[12]. The forest refugium thesis proposed by Haffer[13] suggests that during the last glaciation much of South America consisted of dry savannah, interspersed with "islands" of humid forest in which speciation of many animal groups could have occurred. Based on the present-day habitat preferences seen in *L. longipalpis*, these climatic conditions might have allowed these insects to attain the wide distribution currently seen. As well as deforested and peri-urban settings, *L. longipalpis* occurs in remote forested areas of Amazonian Brazil[14] and Colombian coffee plantations[2].

Both the approaches presented here confirm the fact that *L. longipalpis* is a complex of at least three and probably more species, none of which has been formally described. Further studies are needed to resolve the discrepancies between the two sets of results and permit estimations to be made of the true nature of the complex and the potential for increased transmission of *Le. chagasi* by *L. longipalpis sensu lato* as a result of human modifications of the environment.

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RT4 - *PLASMODIUM* DEVELOPMENT IN THE MOSQUITO MIDGUT: IMPLICATIONS IN DEVELOPING NEW MALARIA-TRANSMISSION BLOCKING STRATEGIES

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Despite concerted international efforts, malaria remains the most devastating insect-borne parasitic disease of humans. The lack of an acceptable protective vaccine and the spread of drug-resistant parasites have made it difficult to control this killer disease, however, its enormous death toll clearly mandates alternative strategies such as transmission-blocking vaccines. Most studies to identify targets for a transmission-blocking vaccine have focused on parasite molecules; however, the complex interaction between the parasite and mosquito molecules — which allow the parasite to develop successfully and transmit — suggest that a vector molecule may also be a potential target that could be exploited to develop novel strategies to control malaria. Here we discuss the factors that influence parasite development in the mosquito and speculate on the targets of potential vaccines for blocking the further development of the parasite.

A *Plasmodium* gametocyte in the lumen of the mosquito midgut encounters a variety of physical and biochemical factors. Two physical factors — a lower temperature and a higher pH — induce gametogenesis *in vitro*; however, it is not clear whether the mosquito midgut pH increases after ingestion of the blood. A gametocyte-activating factor (GAF) that was postulated earlier has recently been purified and characterized. GAF induces *Plasmodium* gametogenesis at physiological pH. The structure of the factor is identical to xanthurenic acid, and synthetic xanthurenic acid induces gametogenesis at nonpermissive pH. In addition to GAF, a variety of midgut factors have been shown to affect *Plasmodium* development. Among these are vertebrate blood factors such as the density of blood cells, soluble RBC factors, leukocytes, antibodies, and complement proteins. The mosquito peritrophic matrix and the proteases released to digest the ingested blood may also act as barriers to parasite development, whereas parasite chitinase and protease-resistant surface coat may allow the parasite to overcome these barriers. The bacterial flora in the mosquito gut, which amplify several folds after ingestion of blood, also affect the normal development of the parasite in the mosquito gut.

The ookinetes that develop successfully from zygote to motile ookinete in the midgut lumen must recognize and invade the midgut epithelium. Mosquito midgut is composed of a complex monolayer of various epithelial cells. These cells serve different functions in the midgut, e.g., columnar cells are involved in the digestion and ingestion of blood, and endocrine cells secrete various hormones. *Plasmodium* interaction with this complex tissue is not clearly understood.

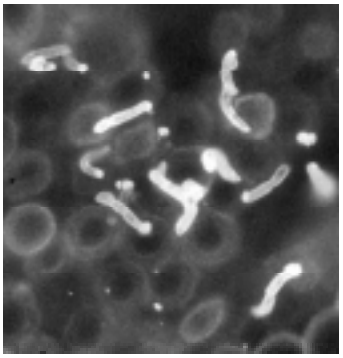
We have developed a series of complementary *in vitro* techniques to study ookinete interactions with the mosquito midgut epithelium. We prepared sheets of midgut epithelium from blood-fed mosquitoes mixed with *in vitro*-transformed *P. gallinaceum* ookinetes. The ookinetes bind to the midgut epithelium specifically and invade with high efficiency (Fig.1). Using these techniques, we found that the *Plasmodium* ookinetes recognize the midgut epithelium via a carbohydrate ligand that covers most, if not the entire, luminal surface of the midgut. The ligand shares several properties of sialic acid; however, an extensive search to identify the sialic acid was not successful. This suggested that the mosquitoes synthesize novel carbohydrates on their midgut surface that mimic the properties of sialic acid and that the *Plasmodium* ookinetes have evolved to use the ligand to recognize the target tissue it needs to invade for further development.

The process of invasion of mosquito midgut epithelium is less well understood than is the invasion of RBC by merozoites or hepatocyte by sporozoites. Using *in vitro* and *in vivo* procedures, we have closely examined the process of the midgut invasion. Electron microscopic and light microscopic studies of the parasites and the invaded cells reveal several novel features of the invasion process. Contrary to earlier ambiguous observations, we found that *P. gallinaceum* ookinetes invade *Aedes aegypti* midgut through the intracellular route.

The most remarkable feature of the ookinete invasion was that more than 95% of the invading ookinetes were found in cells that stained poorly with toluidine blue in histochemical sections. Toluidine blue stains cells that are basophilic, such as columnar cells of the mosquito gut. This suggested that the *Plasmodium* ookinetes preferentially avoid columnar cells when invading. In some cases, as many as five parasites invaded one lightly stained cell. Ultrastructural studies showed several unique features of these specialized cells: they have few or no microvilli on the lumen side; they have large number of vacuoles near the apical end; and they have tubular extensions from the basal side. The cells also express a large amount of vesicular ATPase relative to other cells in the posterior midgut epithelium. We named these novel cells, which are preferentially invaded by the *Plasmodium* ookinetes, "Ross cells."

The parasites that successfully cross the midgut epithelium and settle between the basal lamina and midgut cells are exposed to the rapidly changing biochemical environment and immune molecules in the hemocoel. Malaria vector mosquitoes are anautogenous – they need a blood meal for egg development. Metabolites from the blood modify the hemolymph composition, and the developing parasite is exposed to these biochemicals. In addition, the parasite is also exposed to the immune factors that are expressed in response to the invasion of pathogens that may invade the insect with the blood meal.

The recognition of the midgut tissue via a specific receptor and invasion of a specialized cell implies that both the mosquito molecules and the parasite molecules involved in the processes are potential candidates for modification for blocking the transmission of malaria. Factors that help or retard the development of the parasite in mosquitoes are also important considerations in designing strategies to block malaria.



Adhesion of *Plasmodium gallinaceum* ookinetes (bright, elongated shapes) to the lumen of *Aedes aegypti* posterior midgut tissue (the round background structures are individual mosquito gut epithelial cells). *In vitro* transformed ookinetes were stained with fluorescent dye, PKH26 for visualization. Stained parasites were mixed with isolated mosquito midgut sheets prepared 24 hr after a blood meal. The midgut sheets were labeled with a fluorescent-tagged lectin for visualization.

RT4 - POPULATION GENETICS AND THE NATURAL HISTORY OF DOMESTICATION IN TRIATOMINAE

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The use of isoenzyme electrophoresis applied to population genetics studies of Triatominae was initiated in the eighties (Tibayrenc et al. 1980), remaining now-a-days an appreciable tool for combing population and taxonomic studies. It could generate durable hypothesis about the age of domestic populations of *Triatoma infestans* and its probable geographic origin (Dujardin 1990, 1998a). Together with the use of different markers, it brings now the bases for a predictive model describing the natural history of species adapting to domestic ecotopes.

Species homogeneity of various Bolivian domestic and silvatic populations of *Triatoma infestans* (Klug) had been assessed by an individual examination of isoenzymatic variation at 19 loci (Dujardin & Tibayrenc 1985a, b, Dujardin et al. 1987). On a larger sample including Bolivia, Brazil, Peru and Uruguay, it had been shown that the species was structured into small units among which poor gene flow seemed to occur, and that geographic distances rather than selective factors could account for a significant component of the genetic heterogeneity (Dujardin et al. 1990, 1998a). Whether the genetic variability of *T. infestans* was lower than usual for an insect remained a controversial issue, since different electrophoretic techniques diverged in their estimates of heterozygosity (Garcia et al. 1995b, Dujardin et al. 1998a). These techniques agreed however on a relatively lower variability of *T. infestans* when compared to silvatic species such as *T. rubrovaria* and *T. platensis* (Pereira et al. 1996), *T. sordida* and *T. guasayana* (Garcia et al. 1995a, Noireau et al. 1998), or *T. spinolai* (Frias & Kattan 1989).

More important was the disclosing of very low genetic distances among natural populations of the Southern Cone countries (Garcia et al. 1995[a], Dujardin et al. 1998a), suggesting a recent spread of the domestic populations of *T. infestans* (Dujardin & Tibayrenc 1985b, Dujardin et al. 1998a). An important epidemiological consequence of such a genetic similarity overall was the probability of a similar behavior in response to the same control measures

in different geographic areas. On the other hand, a relatively lower isoenzymatic variability could also suggest a reduced genetic repertoire lowing the probability of insecticide resistance (Schofield 1988).

The genetic structure of the same set of data (Dujardin et al. 1998a) seemed to support also the notion of a unique, ancestral population. This hypothesis was investigated assuming that the population dispersal of *T. infestans* had produced random dispersal of different genes - in which case the ancestral population is expected to present similar gene frequencies to those found in the whole sample. On this basis, we could incriminate Bolivia as the possible geographic origin of the domestic populations of *T. infestans* (Dujardin 1990, Dujardin et al. 1998a). This was in accord with the existence of true silvatic colonies of *T. infestans* only known from the Cochabamba region of Bolivia (Torrico et al. 1947, Dujardin et al. 1987). It was also in agreement with historical records suggesting *T. infestans* had entered to into Peru, Uruguay and Brazil at around the turn of this century (Lumbreras 1972, Osimani 1937, Schofield 1988).

Thus, the hypothesis of a recent and rapid spread of *T. infestans* from one geographic source could accord with population genetics, epidemiological and historical criteria. It was also indirectly supported by the existence of similar geographic clines disclosed from the supposed origin (Bolivia) to more peripheral areas, as revealed by different techniques. From Bolivia to Uruguay, cytologic analyses showed a decrease of C-banding variation, as well as a striking decrease of DNA content in the gonads of male *T. infestans* (Panzera, pers. comm). Between the same countries, head morphometrics showed a consistent cline of decreasing size (Dujardin 1998b).

Similar features were found in *Rhodnius prolixus* (Stål), a highly adapted domestic species and principal vector of Chagas disease in Venezuela, Colombia and parts of Central America. Isoenzyme surveys of natural populations of this species also were consistent with poor genetic variability (Harry et al. 1992, Dujardin et al. 1998b). Further RAPD and morphometric data on *R. prolixus* samples from Honduras and Colombia suggested that this species in Central America could represent a recent and genetically limited subset of the original South American populations, probably those of Venezuela (Dujardin et al. 1998b). This was in accord with historical records (Zeledón, see Schofield & Dujardin 1997) suggesting that *R. prolixus* in Central America was derived from an accidental escape of laboratory bred bugs in 1915, which were subsequently transported in association with people visiting different rural areas. Recent genetic comparisons also supported the hypothesis of immigrated domestic populations of *R. prolixus* in Colombia (Tolima) (Chavez et al., unpub. data). In this way, *R. prolixus* would have invaded several countries as a domestic species, in much the same way that the related *T. infestans* colonized the Southern Cone countries during the last 100 years (Schofield 1988).

Altogether, these observations outline a general model applying to Triatominae in the process of domestication, model in which loss of ecological plasticity and spread of the insect combine their effects to produce an obligate domestic habit. Four steps could be described: (a) since only part of the silvatic genotypes may be successful in establishing durable domestic colonies, some restriction of genetic variability is assumed during the early domestication process. (b) The dispersion of the insect becoming dependent of its host, passive spread of adapted insects occurs. Of more epidemiological relevance, the insect is likely to be transported by humans over large geographic distances, out of the current range of its ecological constraints. (c) As a consequence of both isolation from original silvatic foci and founder effects in the new areas of colonization, a further loss of genetic variability is expected. (d) As long as this geographic expansion goes on, domesticity becomes a more exclusive habit, and some populations with high levels of inbreeding may show external evidence of developmental instability, such as increased fluctuating asymmetry or unilateral morphological monstrosities (Dujardin et al. unpub. data). Recently, it has been observed that they also present decrease of global size (Harry 1994, Dujardin et al. 1997, 1998c) or reduced sexual dimorphism (Dujardin et al. unpub. data).

Such populations, like most of the *T. infestans* or *R. prolixus* domestic populations, should be more vulnerable to control measures. However, the dramatic geographic spread seemingly associated with domestication is of concern. It stresses the need for a careful entomological surveillance of those Triatominae who are presently exhibiting trends to domesticity.

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RT4 - CHARACTERIZATION OF MOLECULES OF *TRIATOMA INFESTANS* SALIVA THAT INTERFERE WITH THE BLOOD UPTAKE

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Haemostatic and inflammatory responses of vertebrate host represent two key obstacles for acquisition of blood meal by haemathophagous insects. Haemostasis acts through three interacting systems; the coagulation cascade, platelet aggregation, and vasoconstriction, which avoid the loss of sanguine fluid (1). Inflammation is responsible for mobilizing effectors like neutrophils, mast cells, and endhotelial cells at the injured tissue, in order to eliminate the noxious stimulus. Besides, most inflammatory mediators are vasoactives, and also cause pain (2).

To overcome these responses, salivary glands of bloodsucking insects produce a large variety of molecules capable of antagonizing them. In fact, several anticoagulants, inhibitors of platelet aggregation, vasodilators and scavengers of inflammatory mediators were described and isolated from insect's salivary glands (3).

In this communication we have studied the saliva of *Triatoma infestans*, which is the principal vector in the transmission of Chagas's disease. As expected, we found an anticoagulant activity and a factor inhibiting platelet aggregation in the saliva. Unexpectedly, we found a novel sialidase and proteolytic activities, which were biochemically characterized in this study.

The sialidase is able to hydrolyze specifically sialic acids linked a 2,3 to galactose, which is usually present in the non-reductor termini of several sialoglycoconjugates. The sialidase is released during the insect bite, and could eventually interfere with neutrophils homing at the inflammatory sites, or with mast cell degranulation by the peptidergic pathway, which are mediated by receptors that recognize ligands containing sialic acid (4,5). The sialidase was purified to homogeneity (5000x) and was show to be a 33 kDa protein that it is synthesized in epithelium of *T. infestans* salivary glands.

The presence of sialidase is unique in *Triatoma*. Other insects, including members of Reduvidae genus, such as *Rhodnius*, lack this activity. It is possible that *Triatoma infestans* sialidase might had been acquired by horizontal gene transfer from bacteria, the vertebrate, or eventually from *Trypanosoma*, all of which having sialidases with conserved structural motifs (6). A kinetic analysis of *T. infestans* sialidase demonstrate that it is close to *Salmonella* enzyme, but molecular cloning and sequencing are necessary to further elucidate this evolutionary linkage.

The other unexpected activity found in *T. infestans* saliva is proteolytic activity, which is partially inhibited by soybean trypsin inhibitor, factor Xa inhibitor from *Bauhinia ungulata*, and PMSF. The presence of proteases in the saliva is unusual since inhibitors of serine-protease of the coagulation cascade are common in haemathophagous insects (3). Protease activity is localized in the lumen of the salivary glands as a zymogen and is released as an active enzyme at the moment of the insect bite. The active protease present in the saliva migrates in SDS-PAGE as a 40

kDa duplet protein as seen in gelatin containing gels. At least two different enzymes were purified by Hi-Trap Q, Phenyl-Superose, and Superdex HR75 chromatography, resulting in major 40 kDa bands visualized by silver staining of SDS-PAGE. We are investigating the fine specificity of the purified proteases and their natural targets.

Characterization of these activities may reveal additional mechanisms to avoid the host responses, and be important to develop new targets in vector control. Furthermore, these molecules may be potential pharmacological tools.

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RT5 – ANAEROBIC PROTOZOA

RT5 - PURIFICATION AND IMMUNOCYTOCHEMICAL LOCALIZATION OF NEURAMINIDASE FROM *TRITRICHOMONAS FOETUS*

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Previous studies have provided evidence for the presence of protein with neuraminidase activity secreted by *Tritrichomonas foetus* [1,2,3]. This enzyme was initialy considered to be located in lysosomes [2,3]. However, further studies showed that *T. foetus* spontaneously releases neuraminidase into the growth medium in a soluble form. In addition it was shown that contact of the parasites with erythrocytes led to release of sialic acid residues, thus suggesting the presence of a membrane-bound neuraminidase. Our previous studies showed the presence of a neuraminidase associated to the plasma membrane of *T. foetus* via a glycosylphosphatidylinositol anchor [4].

Our present biochemical studies show that the purified enzyme differs clearly from the two neuraminidases reported by Crampen et al. [3]. The molecular weights of the two neuraminidases, isolated from culture medium, were determined as 320,000 and 38,000. However, desintegration of the largest enzyme by urea yielded a fraction of 79,000, suggesting the enzyme was a tetramer consisting of four subunits of equal size. This finding was supported by the preparative starch gel eletroforesis. Our present observations show that all enzyme activity towards the substrate 4MU-NANA eluted in one peak from a Bio-Silect SEC 250-5 gel permeation column in HPLC at a volume corresponding to a molecular weight of 80,000 as estimated using a least squares plot constructed for a range of protein of known molecular weight. On the other hand, when the single protein band which migrated in the same region as the enzyme activity obtained on native polyacrylamide gel electrophoresis was excised from the non-denaturing gel, and re-runned on denaturing SDS-PAGE, one lightly stained band with apparent molecular weight of 80,000 was found, indicating that the neuraminidase consists of a single unit.

Immunoblott of the purified enzyme incubated with polyclonal antibodies against purified neuraminidase confirm the purity of the enzyme and also revealed a single 80 kDa band. Indirect immunoflurescence microscopy using specific antibodies raised against the purified neuraminidase, indicated that the enzyme is present on the cell surface. This observation was confirmed by experiments showing intense of labeling of the cell surface when living parasites were incubated in the presence of the antibodies. Also, addition of the purified protein to the incubation medium significantly reduced antibody staining. These results confirm the specificity of the antibodies for the neuraminidase expressed on the cell surface of the trichomonads. Similar results were obtained when the antigenic sites were localized by electron microscopy using cryosections incubated first in the presence of anti- neuraminidase antibodies followed by incubation in the presence of gold-labeled anti-rabbit IgG. In addition, labeling of some cytoplasmic vesicles was also observed. it is possible that they correspond to secretory vesicles originated from the endoplasmic reticulum-Golgi complex system in route to the plasma membrane. In view of the fact that labeling was mainly associated to the vesicle membrane rather than in its matrix it is possible that the enzyme following fusion of the vesicle with the plasma membrane the enzyme is inserted into the membrane and not secreted into the medium. Release of the enzyme into the medium could result from the action of an endogenous PIPLC.

Previous reports have indicated that the cytotoxic action of trichomonads is at least in part related to a prior physical contact between the surface of the two interacting cells [5,6]. A possible effect of the trichomonad neuraminidase on the cytotoxic effect exerted by both *Trichomonas vaginalis* and *T. foetus* on epithelial monolayer has been suggested [1]. Further studies using the purified enzyme may clarify basic aspects of the role played by neuramini-

dase on the process of interaction of *T. foetus* with epithelial cells.

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RT5 - THE STUDY OF DYNAMIC PROCESSES IN THE ANAEROBIC PROTOZOAN *TRITRICHOMONAS FOETUS* AND *GIARDIA LAMBLIA*

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The use of advanced systems of video-microscopy and analogue and digital imaging processing devices allowed us to analyze, in detail, some dynamic phenomena related to the biology of anaerobic protozoan, specially *Tritrichomonas foetus* and *Giardia lamblia.* The study of these processes came to be of great importance when we observed that, in order to interact with target cells, these protozoan should dislocated themselves in a turbulent liquid medium.

T. foetus is a flagellated protozoan of the urogenital tract of cattle. Using its flagella, three anterior and one recurrent, the cell swims and interact with epithelial cells. Besides the presence of the flagella, the cell presents stable cytoskeleton structures named Costa and Axostyle, that were believed to present proper motion. By the use of video-microscopy and analogue and digital recording devices we could analyze frame by frame the swimming pattern of *T. foetus*, as well as, the existence (or not) of motion related to the stable cytoskeleton structures. The data obtained lead us to concluded that the four flagella participate in the motion and that the Costa and axostyle do not present proper contractibility. Besides that, we observed a very unique kind of swimming pattern in these cells. All four flagella emerge in a same region, in the anterior portion of the protozoan. In this region we noted that the waves in the flagella always begin in the same time, but the resultant waveform were very distinct in three of the four flagella. Another interesting observation was that the frequency in the three anterior flagella was always half the one observed in the recurrent flagellum, despite a decrease or an increase in the rate itself. That means that should exist some kind of coordinated factor that signalized the begging of the cycles, no matter what kind of waveform will be produced. Another point is the swimming pattern itself. This pattern is always related to the position and direction of the flagellar beating in the cells. In the case of *T. foetus*, the three anterior flagella were positioned forwards, and as we could observe, present a cilia-like beating. On the other hand, the recurrent flagellum originates in the anterior portion, but is positioned towards to the posterior region. As a consequence of these distribution the cell did not present a constant forward dislocation. During part of the beating cycle in the anterior flagella, the cell is pushed backwards by these flagella, but this backwards dislocation is blocked by the opposition of movement of the recurrent flagellum. In the rest of the cycle in the anterior flagella, these structures push liquid backwards, in the same direction of the recurrent, causing a dislocation of the cell in the opposite direction, the forward direction. The cell probably uses the flagella to interact with host cells, but the real participation of these structures were not established yet.

Giardia lamblia is a multiflagellated protozoan of the intestinal tract of humans and is the responsible of giardiasis, an intestinal disease that affects thousands of people all around the world. The cell presents four pairs of flagella: an anterior, a posterior, a ventral and a caudal pair. It also presents a very sophisticate stable cytoskeleton formed by structures composed mainly by tubulin such as the adhesive disk, median body and a microtubular complex. This complex is organized by the intracitoplasmic flagellar axonemes and by microtubular laminas that follow the caudal axonemes in its cytoplasmic portion. As observed in *T. foetus*, *Giardia* also dislocates in the lumen in order to adhere and parasite the epithelial cells. The video and image analysis of *Giardia* revealed that only the anterior and the ventral flagella were responsible for the swimming pattern of the cell. We observed that the posterior and the caudal flagella do not present proper motion. The dislocation of the cell forwards is mainly caused by the beating of the ventral pair, but the anterior pair also participate in this movement and is directly related to the lateral vibration or the rotation of the cell. This last aspect, the lateral vibration or rotation during the forward dislocation was observed in several images. We believe that the occurrence of one situation or the other depends on the position of one of the anterior flagella in relation to the other. As pointed out in a model created by us, if the flagella were in opposition, as would happens if we observe that the cell presents bilateral symmetry, the movement will be a lateral vibration; but if one flagellum, as sometimes happens, positioned itself over the cell body, the resultant vector will be coincident and the cell will start to rotate. We observed the both situation in video-micros-

copy images. After swimming in the intestinal lumen, *Giardia* will adhere to the epithelium using its adhesive disk. The process of adhesion, although studied by some scientists, is still lacking a clear explanation. We analyzed this process using our system, and observed very interesting images. The current belief is that the disk itself do not present contractibility, and the adhesion process is a function of the presence of actin and myosin in the lateral crest. The images obtained by us showed a strong contraction of the disk, and a cyclic contraction, that we believe could only be associated to a stable cytoskeleton structure. We did not observe any stable actin or myosin structure in the electron microscopy analysis. So we do believe that the process of adhesion has the active participation of the adhesive disk itself and we are analyzing this hypothesis in the moment. We also observed an interesting phenomenon in the caudal region of the cell. When the parasite is attached or free in the medium, it presents lateral and/or dorso-ventral movement of its caudal region. We assume that the microtubular complex formed by the caudal axonemes and the microtubular sheets are the responsible for this movements. Indeed, in video-microscopy images, we clearly see that one axoneme rolls over the other during these dislocations. By the use of 3D reconstruction of serial sections, we are trying to understand the exact role of these cytoskeleton structures on these movements. The study of the dynamic phenomena in *Giardia* continues with the observation of the process of interaction between the parasite and a epithelial monolayer *in vitro*. Using IEC-6 (Intestinal epithelial cell line) we are trying to follow the attachment and detachment process, as well as, the possible participation of the flagella and the microtubular complex in the interaction process. We are also using some anti-helmintic drugs as benzimidazole, albendazole, mebendazole, that works also against giardiasis by video-microscopy assays. These drugs are been studied against *Giardia* because its main target is tubulin, the major protein in *Giardia* cytoskeleton. After the acquisition of a lot of data about dynamic processes of *Giardia*, including motility, adhesion and cell body movements, we are investigating the effects of the above drugs in these phenomena. We observed that some drugs cause a massive destruction in the ventro-lateral flange and a decrease in the flagellar beating frequency, but our preliminary results show that only when the structure of the adhesive disk is affected that the cell begins to detach. All the process were followed by video-microscopy and afterwards the structural damage caused by the drugs were accessed using the transmission electron microscopy. The effects of the anti-helmintic drugs will be also investigated in the IEC-6, in order to analyse the possible damage of these chemicals to the epithelial cells.

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RT5 - A CYSTEINE PROTEINASE OF 65 KDA (PC65) PARTICIPATES IN *TRICHOMONAS VAGINALIS* CYTOTOXICITY

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Trichomonas vaginalis is a sexually transmitted protozoan parasite of the urogenital tract of humans. Significant suffering among women results from infection, colonization and cytopathogenicity by this microorganism. Whether the cytopathic effect of *T. vaginalis* infection are induced by metabolic products released by the parasites or are caused by direct contact between the parasite and epithelial cells or a combination of both mechanisms are at present unknown. Also, the molecules involved in cellular damage remain poorly defined. Trichomonads have multiples proteinases mainly of the the cysteine proteinase type, these molecules have received attention for their possible role in virulence properties i.e., cytoadherence, cytotoxicity, haemolysys, nutrient acquisition, immune evasion, etc. (2). Recently, we have identified and characterized a 65 kDa cysteine proteinase (CP65) involved in trichomonal cytoxicity. It is localized on the surface of *T. vaginalis* and has affinity to the surfaces (3,4). Specific polyclonal antibodies to CP65 and cysteine proteinase inhibitors (CPI) i.e., , E 64 and antipain used in cytotoxicity assays and substrate gel electrophoresis had helped to demonstrate its participation in cytotoxicity. The anti-CP65 antibody that reacted with the parasite surface by indirect immunofluorescence inhibited parasite cellular damage in a concentration dependent manner up to a 64%. Both E-64 and antipain abolished CP65 activity and inhibited trichomonad cytotoxicity up a 45% without affecting parasite viability nor motility. The CP65 proteinase activity has an optimum pH of 5.5 at 37 C, pH found in patients with trichomonosis, and a pl of 7.2. Also, CP65 proteinase and antibodies to CP65 were found in vaginal secretions and patient sera. These data show the in vivo relevance of class of molecules as well as its potential for future drug targeting development.

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RT5 - FOCAL ADHESION IN *TRICHOMONAS VAGINALIS*

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The human parasite *Trichomonas vaginalis* may be found associated to the epithelium lining urogenital cavities. Studies foccusing the interaction process between *T. vaginalis* and host epithelial cells in *in vitro* conditions seems to reproduce most of what is observed in the parasite-harboring patients. By using monolayers formed by epithelial cells many investigators have identified parasite surface molecules involved in each one of cytoadhesion and cytotoxicity steps. It has been shown that during the cytoadhesion step parasitic surface molecules named adhesins are directly involved in the phenomenum while parasitic proteinases belonging the cystein family seems to be responsible for most of the cytotoxicity exerted by trichomonads to the epithelium. Of interest is the fact that much of the patients suffering urogenital trichomoniasis present lesions in both ectocervix and endocervix at high extent. Some questions emerge from these observations. One of them which is very interesting to investigate is concerning the possibility of *T. vaginalis* to be able to induce lesions in uterine colon and/or to invade uterus.

Immunohistopathologycal assays were carried out by using biopsies of uterine colon from women suffering trichomoniasis. Most of the assays did result in a intense reaction of the epithelial cells when polyclonal antibodies anti-human laminin (isoforms 1 and 5) were assayed. Intense reaction was observed during assays carried out with antibody anti-laminin 1. This isoform 1 of laminin which is also known as laminin from EHS tumors is represented by a 900 kDa protein whose aminoacid sequences IKVAV, RGD, and YIGSR may be recognized by some procaryotes and eucaryotes. On the other hand, *T. vaginalis* can recognize laminin-1 through a parasite surface molecule of 118kDa. Experiments designed to identify the minimal aminoacid sequence of laminin-1 recognized by *T. vaginalis* resulted that YIGSR is the preferential residing adhesion sequence for this trichomonad.

Observations of parasites adhering on laminin-1-coated substrata revealed that all microorganisms change their morphologies resulting in high polymorphism. Most of the observed laminin-1-contacting microorganisms change their morphologies from “tear” forms to aberrant forms. The last ones show cytoplasmic contacts resembling focal adhesion processes.

The possibility of *T. vaginalis* to form focal adhesion contacts as soon as it is found contacting laminin-1 is the subject of our present investigation. If the “aberrant” forms of *T. vaginalis* are the result of focal contacts established between parasite and laminin-1 it is reasonable to infer that parasite must possess (a) integrins or (b) laminin-binding proteins on its surface, and (c) proteins intracellularly associated to such integrins or laminin-binding proteins. That is to say, laminin-1 could be a signal for the parasite to change its morphology, and became it highly cytoadhesive to epithelial cells.

In terms of human trichomoniasis this is a subject of relevance since the usual ameboid forms of *T. vaginalis* which have mainly been observed in cronic cases of the disease could represent the response of the parasite to its interaction with laminin-1 as well as with other ligands presenting each one of the aminoacid sequences RGD, YIGSR, and IKVAV.

By using columms made of laminin-1 coupled to Sepharose 4B we were able to obtain parasite molecules which bind to laminin-1. Initial identification of these molecular species from the parasite were made in SDS-PAGE. A proeminent band of 118-120kDa was obtained which in turn, during immunoblotting assays reacted with each one of monoclonal antibodies anti alpha 3 beta 1 chains of integrins. Since integrins did not contain phosphorylation sites which could provide “activation” of closely associated cytoplasmic molecules we decide to investigate the occurence of pp 125 FAK in the parasite. Monoclonal antibodies anti-human pp 125 FAK strongly reacted with cytoplasmic proteins of *T. vaginalis*. Further, experiments carried out by using antibodies anti-paxillin and anti-Src in co-immunoprecipitation assays revealed that the (a) laminin-1 can induce cell signalling in *T. vaginalis*, and that (b) pp 125 FAK, paxillin, and Src are involved in such cell signalling process.

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RT6 – IMMUNOLOGY IN PROTOZOAN DISEASES

RT6 - SOLID IMMUNITY AFTER SINGLE DOSE VACCINATION IN A PRIMATE MODEL OF CUTANEOUS LEISHMANIASIS

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Protection from the morbidity of cutaneous leishmaniasis has been achieved in humans only by injection with live preparations of the parasite. This practice has been abandoned in most areas due to problems with consistent

manufacture and storage of live vaccine, as well as the occasional severe local reaction. We used *Rhesus* macaques to assess the safety, immunogenicity, and efficacy of a vaccine that combines a crude heat killed preparation of *Leishmania amazonensis* (LeishVaccin, Bíobras, Brazil) with recombinant human interleukin-12 (IL-12, Genetics Institute) and aluminum hydroxide fluid gel (Rehydragel HPA alum, Reheis) as adjuvants. A single subcutaneous injection in the arm was used for vaccination in forty-eight monkeys. This combination was found to be safe and immunogenic, although a small transient subcutaneous nodule developed at the site of vaccination. Groups receiving IL-12 had an augmented *in vitro* antigen specific IFNgresponse for two to four weeks after vaccination, as well as an increased production of IgG antibodies, in a dose dependant manner. No IL-4 or IL-10 was present in cultures of peripheral blood mononuclear cells from either control or experimental groups. Intradermal forehead challenge infection with 10^7 metacyclic *L. amazonensis* promastigotes at four weeks demonstrated solid immunity in all twelve monkeys receiving 2µg IL-12 and at all antigen/alum doses as a result of vaccination. Partial efficacy was seen with lower doses of IL-12 and in the groups lacking either adjuvant. All but one of the sixteen monkeys in the control groups developed a typical cutaneous lesion.

Similar results were seen in a parallel experiment in another twelve monkeys with a single intradermal injection in the small of the back using a crude *L. major* killed antigen (Razi Institute, Iran) with low dose BCG vaccine and alum as adjuvants. An ulcerative nodule developed locally that healed after several months. Antigen-specific production of IFNgwas increased by one month after immunization. Challenge infection with *L. amazonensis* at four months after vaccination showed protection in nine of ten monkeys receiving both adjuvants, also demonstrating cross-species efficacy in the model. Further experiments are being done to study and minimize nodule formation in both systems. Thus, a single dose vaccination with killed crude antigen using IL-12 or BCG in combination with alum as adjuvants was safe and fully effective in this primate model of cutaneous leishmaniasis. Careful assessment of safety and efficacy will be required to determine if the same is true for humans at risk of developing cutaneous disease.

RT6 - DNA VACCINATION AGAINST LEISHMANIASIS

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Most currently licensed vaccines for humans induce protective immunity via the induction of neutralizing antibody. Importantly, these responses are maintained over a long period of time. For certain pathogens (eg *Mycobacterium tuberculosis, Leishmania* sp., ? HIV), cellular immune responses are required for protection. The kinds of antigens or adjuvants that might optimize the long term maintenance of cellular responses are not well understood. The murine model of *L. major* infection has provided valuable insight into the role of cellular immunity in mediating protection against an intracellular pathogen. Effective primary immunity to *L. major* infection is due to the development of a Type 1 immune response characterized by the IL-12-dependent production of IFN-g from NK cells and MHC class II-restricted $CD4^+$ T cells (1). In the first demonstration that IL-12 can be an effective Th1 inducing adjuvant in combination with protein antigens, effective vaccination against *L. major* in susceptible strains was achieved using rIL-12 with soluble leishmanial antigen (2), and subsequently with a recombinant leishmanial protein LACK (3). A recent study showed that subcutaneous administration of plasmid DNA encoding the LACK antigen, in the absence of any additional adjuvant, was sufficient to confer protection in an IL-12 and CD8+ T cell dependent manner (4). In all these models, infectious challenge was done within 2 weeks after the second vaccination. We have been attempting to optimize the nature of the leishmania antigens and/or IL-12 adjuvant that are required for induction of sustained cellular immunity. In the study reported here, we compare the ability of antigen specific DNA, or protein antigens, including a whole cell killed *L. major* vaccine (ALM) currently in use in clinical trials, in combination with IL-12 protein or IL-12 DNA, to elicit protective immunity to infectious challenge at least 12 weeks post vaccination.

BALB/c mice vaccinated with LACK DNA or LACK protein + rIL-12 contained lesion growth compared with control DNA when challenged 2 weeks after infection. In contrast, mice vaccinated with LACK DNA but not with LACK protein + rIL-12 were still able to control infection when challenged 12 weeks after the second vaccination. Potential reasons for the failure of LACK protein + rIL-12 to elicit long-term immunity could be due to the inability of responses directed against a single antigen to provide long lasting immunity, and/or to the inability of rIL-12 protein to generate a sustained Th1 response in this model. To address these issues, a clinical grade preparation of heat-killed *L. major* (ALM) was used to provide a wider spectrum of antigens than LACK protein. In addition, IL-12 was administered as plasmid DNA rather than protein to see whether it would provide a more sustained immune response when coadministered with leishmanial protein. Vaccination with LACK DNA, ALM + rIL-12 or ALM + IL-12 DNA was effective in controlling infection when mice were challenged 2 weeks post vaccination. When mice were challenged at 12 weeks post vaccination in the opposite footpad to which vaccination occurred, only mice vaccinated with LACK DNA and ALM + IL-12 DNA, but not ALM + control DNA or ALM + rIL-12 were able to control infection. Thus, vaccination with crude, heat-killed leishmanial antigen can provide durable and protective immunity if IL-12 is provided in the form of DNA rather than protein.

IFN-γ production was assessed from draining lymph nodes of mice infected 2 and 12 weeks after vaccination. Production of IFN-γ from mice infected 2 weeks post vaccination was similar from cells of mice vaccinated with LACK DNA, ALM + IL-12 DNA, or ALM + IL-12 protein. By contrast, production of IFN-γ from mice infected 12 weeks following vaccination was detected only in mice injected with LACK DNA or ALM + IL-12 DNA. In addition, there was a 3- to 4-fold reduction in production of IFN-γ from mice vaccinated with LACK DNA or ALM + IL-12 DNA when anti-class II was added to the cultures. The presence of anti-CD8 mAb also caused a decrease in IFN-γ from cells of LACK-vaccinated mice but not from cells of mice vaccinated with ALM + IL-12 DNA. These data underscore the fact that protein antigens such as ALM or LACK combined with IL-12 or IL-12 DNA induce IFN-γ from $CD4^+$ T cells, while antigen expressing plasmid DNA induces IFN-γ from both $CD4^+$ and $CD8^+$ T cells.

We believe that these data provide strong evidence that durable cellular immunity to protein antigens requires the persistence of IL-12. With regard to the effectiveness of LACK DNA, it is possible that long lived, IL-12 independent CD8+ T cells are elicited by this vaccine that contribute to the sustained immunity. Nonetheless, since plasmid DNA also contains potent immunostimulatory sequences able to induce Type 1 immune responses (5), it is likely that persistence of IL-12 is also maintained by the LACK DNA. The ability to elicit both sustained CD8+ T cell responses and persistent production of IL-12, suggests that antigen expressing plasmid DNA will provide especially potent vaccination against *Leishmania* and other intracellular pathogens.

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RT6 - MAPPING OF T CELL EPITOPES IN RECOMBINANT PFR PROTEINS OF *TRYPANOSOMA CRUZI*

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We have previously shown that immunization of mice with a preparation of purified paraflagellar rod (PFR) proteins from *Trypanosoma cruzi* can provide protection against a subsequent challenge with this parasite. Protection involves both CD4+ and CD8+ T cells, however B cell function is not required. Our findings also indicate that protective immunity in mice immunized with PFR is associated with a Th-1 type response. Furthermore, macrophages infected with *T. cruzi* or cultured in the presence of PFR protein can be activated to produce nitric oxide (NO) by CD4+ T cells from PFR immunized mice, suggesting that macrophage induced parasiticidal activity is an important mechanism in PFR mediated protection. To further investigate the involvement of individual PFR proteins in this mechanism, the complexity of the PFR protein preparation was analyzed. We now have shown that the PFR protein preparation contains four distinct proteins. Analysis of the conceptual proteins encoded by these proteins reveals that two of the proteins, designated PAR 2 and PAR 3, share high sequence homology. The remaining two proteins, designated PAR 1 and PAR 4, share less than 15% a.a. sequence similarity with each other, or with PAR 2 and PAR 3. The genes encoding these proteins have been isolated, sequenced and expressed in both eukaryotic and procaryotic host/vector systems. Recombinant PFR protein from these genes have been found to induce IC-21 macrophage cells to produce NO when incubated in the presence of CD4+ T cells isolated from PFR immunized C57BL/6 mice. To map the epitope(s) present in two of these proteins, designated rPAR 1 and rPAR 2, the genes encoding these proteins have been divided into six regions which overlap by 30-36 nucleotides. These fragments have been cloned into the *Escherichia coli* vector pTrcHis. The polypeptide fragments encoded by these regions have been isolated and their ability to induce IC-21 macrophages to produce NO in the presence of T cells from PFR immunized mice has been determined. The results indicate the presence of mulitple epitopes in both proteins. Using sets of polypeptides of length 15 a.a. that overlap at 3 a.a. intervals, these epitopes have been mapped at high resolution.To date, studies to investigate the immunologic mechanisms of PAR-mediated protection have been performed using Freund's adjuvant. However, recent studies using a combination of Alum, recombinant IL-12 or replication-deficient adenovirus containing the IL-12 subunit genes indicate that this adjuvant system may induce a protective immune response in mice similar to that observed with Freund's adjuvant. T cells from mice immunized with PAR, Alum and recombinant IL-12 induce IC-21 macrophages infected with T. cruzi to produce levels of NO similar to that seen with Freund's adjuvant and replication of the parasite is drastically reduced. Moreover, recogni-

tion of infected macrophages by the immunized T cells appears not to be parasite strain restricted, since this effect is seen using T cell from mice immunized with PFR proteins of the Peru strain and macrophages infected with the Esmeraldo 3 clone, Y strain, and CL strain.

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RT7 – GENOME INFORMATION FOR VACCINE AND DRUG DEVELOPMENT

RT7 - NOVEL APPROACHES TO STUDY VIRULENCE GENES IN *LEISHMANIA*

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My laboratory is undertaking research toward identifying andcharacterising virulence genes in *Leishmania donovani*. A major goal of this work is to genetically engineer live attenuated strains in which virulence genes have been removed and to also determine whether recombinant virulence gene products themselves are protective against infection. Our major focusis on the A2 gene family which encodes a family of proteins with repeated sequences which are specifically expressed in the amastigote stage of thelife cycle (1,2,3). Our rational is that proteins specifically expressedin the amastigote stage of the life cycle such as A2, may play a central role in the survival of *L. donovani* in the human host. In order to verify that A2 proteins are required for survival in the mammalian host, it was necessary to remove them and determine the effect on parasite viability *in vivo* (4).

Traditionally, the standard approach for characterising gene function in *Leishmania* has been to perform diploid gene knockouts. Although this approach works well, it is time consuming and in the case of multigene families is often difficult to carry out. This is particularly evident in the case of genes which are tandemly repeated with other genes within the same DNA sequence. This is the case for the A2 gene family where the A2 genes are tandemly repeated with an unrelated gene which we have termed A2rel (2). It was therefore necessary to attempt a more rapid and simple approach for targeting A2 gene expression in *Leishmania* cells.

One method which has been used in higher eukaryotic cells isantisense RNA which can specifically hybridise to the sense mRNA which results in the destabilisation of the corresponding mRNA. We have therefore attempted to use antisense RNA to inhibit the production of A2 protein in *L. donovani* (4). Our strategy was to overexpress the A2 antisenseRNA specifically in the amastigote stage using a plasmid construct containing A2 derived regulatory sequences which stabilize RNA underamastigote conditions. In this manner, it was possible to obtain amastigotes which had high levels of A2 antisense RNA relative to A2 mRNAand under these conditions this resulted in the destabilisation of the A2mRNA and a concomitant loss in the A2 protein from the amastigotes.

Characterization of the antisense RNA containing A2 deficient *L. donovani* revealed some interesting results (4). First, A2 deficient cells could multiply in culture as efficient as wildtype cells both aspromastigotes or amastigotes revealing that A2 was not required for survival in culture. Second, A2 deficient amastigotes were also able to proliferate inside macrophages, although not as efficiently as wildtype amastigotes. Third, A2 deficient amastigotes were severely attenuated in their ability to survive in Balb/C mice relative to wildtype amastigotes. Interestingly, the amastigotes which were recovered for the liver of the mice had retained the ability to express A2 indicating that in the mouse model, there was a selection against the amastigotes which had lost the ability to express A2. These data strongly argued that A2 protein can be considered as a virulence factor required for survival in the mammalian host and this provides strong justification for carrying out the A2 gene knockout experiments which are currently underway.

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RT8 – PROTEASOME IN PROTOZOA

RT8 - ROLE OF PROTEASOME IN THE LIFE CYCLE OF *TRYPANOSOMA CRUZI*

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Trypanosoma cruzi undergoes profound morphological changes during development in the vertebrate and invertebrate hosts. When trypomastigotes invade host cells and enter the cytoplasm, they transform into amastigotes.

The amastigotes replicate and within several days they transform back into trypomastigotes. During this time *T. cruzi* undergoes shape changes, restructures its flagellum and its kinetoplast, and synthesizes different sets of surface molecules. When trypomastigotes are incubated with lactacystin, a specific inhibitor of the 20S proteasome, trypomastigotes fail to transform both in culture medium and within cells. In this report we further characterize the properties of the *T. cruzi* proteasomes, and attempt to document the participation of the ATP-dependent ubiquitin-pathway in the proteolysis that occurs during trypomastigote transformation into amastigotes.

RT8 - ARE THE PROTEASOME GENES CLUSTERED IN THE *TRYPANOSOMA CRUZI* GENOME?

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The proteasome, a multimeric, ATP-dependent multicatalytic proteinase playsan essential role in the non-lysosomal degradation of intracellularproteins. Among the substrates which are degraded by the ubiquitin pathwayare regulatory proteins such as cyclins (Ghisiain et al. 1993, Wojcik et al. 1996), the products of some oncogens (Tsurumi et al. 1995), p53 tumor suppressor factor (Scheffner et al. 1990) and components of the nuclear factor-kB transcriptional complex (Palombella et al. 1994). Beyond this the complex is also involved in peptide processing for antigen presentation by MHC class Imolecules (Gaczynska et al. 1993, Yang et al. 1995, Groettrup et al. 1996) which makes intracellular parasites and viruses a target for the proteasome (Driscoll & Finley 1992). However while much has been learned about physical structure and biochemical properties of the proteasome from high organisms its roles in specific biological processes such as parasites infection is still very limited.

The 26S proteasome is formed by a 20S proteolytic core and two polar 19S regulatory complexes.The eukaryotic 20S proteasome is composed of 28 different protein subunits, ranging in size over 21-35 kDa, that are assembled in four stacked rings, each containing seven subunits. All sequences of cloned proteasome subunits from diverse organisms can be grouped in two major families based on the homology to either the alpha or beta subunit of the structurally less complex archaebacterial proteasome. The alpha-type subunits are exclusively localized in the two outer rings, whereas the two inner rings are formed by beta-type subunits (Coux et al. 1994).

The recent characterization of the 20Sproteasome from trypanosomatids and comparison with rat proteasome has shown that though they show structural similarities they are functionally dissimilar (Hua et al. 1996). Thus it would be interesting to investigate in detail the protein subunit composition of trypanosomes proteasome and compare it with the proteasome from other organisms. Despite the high number of cloned proteasome sequences in different organisms, cloning of proteasomal genes in trypanosomatids are still in progress.

In order to learn about the structure and the biological function of 20S-proteasome in low eukaryotic systems a genetic and molecular study of proteasome in *T. cruzi* as well as host proteasome upon *T. cruzi* infection has been undertaken in our lab. Previously, a systematic analysis of this particle has been made in various species of trypanosomatids. We have shown that trypanosomatids proteasomes are relatively simple as compared with its mammalian counterpart. Other previous work in our lab led to the cloning of one cDNA family encoding a *T. cruzi* proteasomal alpha type subunit. Here, we show the characterization of their genomic clone termed tcpr29A. In addition, a second proteasome gene has been found in tcpr29A clone. This gene encodes a protein, which is 65% identical to the rat and human beta-type LMPX subunit. Both alpha and beta types subunits are constitutively expressed during the parasite life cycle. Southern analysis and hybridization of labeled tcpr29A genomic sequences to chromosome preparations reveals a single locus on the chromosome XX of CL Brener. These results suggest that proteasome genes could be organized in a cluster in the *T. cruzi* genome.

Another topic of interest in our lab concernsthe proteasome expression in host cells upon *T. cruzi* infection. We have performed these experiment in the presence of gamma-interferon and our preliminary results will be also presented.

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RT9- THE FLAGELLUM OF PROTOZOA

RT9 - COMPUTER MODELLING OF THE CILIARY AXONEME: FUNCTIONAL IMPLICATIONS

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This presentation summarizes work reported by Sugrue et al. (1991) and Holwill et al. (1995). The 9+2 ciliary axoneme of *Tetrahymena* has been reconstructed by computer modelling at a resolution approaching 4 nm. The modelling has utilized the software package SURREAL to represent structures in three-dimensions. The model may be viewed as specified by an observer for direct comparison to electron micrographs of ciliary structure, and it may be modified as new structural or biochemical data is obtained.

Recently, we have successfully reconciled several different interpretations of inner dynein arm (IDA) structure, based on different preparations and microscopic techniques. Current work is designed to study the mechanism of ciliary movement with the model. To this end, it is necessary to examine how an ensemble of dynein molecular motors produces sliding. We have chosen to model the sliding behavior of two microtubules driven by 22S dynein, the outer arm dynein (ODA) of *Paramecium* or *Tetrahymena*. At beat frequencies of ca. 25 Hz, an effective stroke is completed in about 10 msec at microtubule sliding velocities of about 10 mm/sec. (10 mm/msec). About 12 ODA steps in total are sufficient to produce this velocity, which suggests that ODA activity during the effective stroke is stochastic. A stochastic model of dynein arm activity is compatible with the "switch point" hypothesis of ciliary motility, and with the experimental data on microtubule gliding generated by Hamasaki et al. (1995).

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RT9 - BIOCHEMICAL ANALYSIS OF A PURIFIED PARAFLAGELLAR ROD FRACTION

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The flagellum of Trypanosomatids, as well as of Euglenoids, has, alongside the axoneme, a filamentous lattice-like structure which has been designated as paraxial or paraflagellar rod (Anderson & Ellis 1965, Fuge 1969, deSouza & Souto-Padrón 1980). Its function is still unknown, one can only suppose it might play a role in flagellar beating for cell swimming or in cell adhesion (reviewed in Bastin et al. 1996), although trypanosomatids devoid of paraxial rod, such as *Crithidia deanei*, have the same flagellar beating pattern. Important data were recently obtained either by knockout of one of the major constituents of the *Leishmania* rod (Santrich et al. 1997) or by molecular ablation using antisense RNA in *Trypanosoma brucei* (Bastin et al. 1998), rendering the protozoa imotile. Immunologically, paraflagellar rod proteins are very relevant, as several antibodies were raised against them (reviewed in Bastin et al. 1996). When used in immunoprotection tests, they elicited a potent cellular response (Miller et al. 1997).

Ultrastructural analysis revealed that the paraxial rod of trypanosomatids is a complex array of filaments of at least three different thicknesses, arranged in three distinct regions (Farina et al. 1986): (a) one proximal, with two plates formed by 25nm- and 7 nm-thick filaments disposed at 100^0 angle, (b) one distal, with 11 plates with the same aspect of proximal plates, and (c) an intermediate region, less electron-dense, formed by thin filaments that connect the proximal with the distal plates. The whole structure has a fixed position relative to the axoneme, linked to peripheral doublets 4-7 by two types of alternate filaments: one single and other Y or V shaped, periodically positioned between rod and axoneme. Analysis of protein composition of the paraflagellar structure has revealed two major bands of 76-68 kDa in *Crithidia fasciculata* (Russel et al. 1983), 78-73 kDa in *Herpetomonas megaseliae* (Cunha et al. 1984), 75-72 kDa in *Trypanosoma brucei* (Gallo & Schrevel 1985), 70-68 kDa in *Trypanosoma cruzi* (Saborio et al. 1989) and 74-69 kDa in *Leishmania amazonensis* (Ismach et al. 1989). Paraxial rod major proteins are quite similar, presenting high degree of sequence homology and common epitopes (Deflorin et al. 1994).

It seems unlikely that such a complex structure could be formed by so few proteins. In order to address this question, we decided to look for minor proteins that could play a role in the assembly and stabilization of the rod lattice. It would be invaluable to obtain a subcellular fraction containing the paraflagellar rod separated from the axoneme. *C. fasciculata* major paraflagellar constituents were analyzed separated from the axoneme (Russel et al. 1983), but that protocol led to rod solubilization. We have pursued a method that allowed the purification of the

intact paraxial structure. It was achieved using a flagellum isolation protocol (Cunha et al. 1984) followed by demembranation and carefully limited proteolysis (Moreira-Leite et al. 1998, this issue). Axoneme and paraxial rod were torn apart and separated in a sucrose gradient. The electron microscopic aspect of the purified paraflagellar rod fraction was very uniform, showing that paraflagellar rod sub-structure was preserved, with distinguishable proximal, intermediate and distal regions.

Preliminary analysis of the purified paraflagellar rod fraction using uni-dimensional gel electrophoresis identified, besides the major proteins, some minor bands corresponding to 202, 190, 123, 84 and 77 kDa. Additional biochemical and ultrastructural studies are in progress, to further characterize these proteins and localize them in the assembly.

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RT9 - STRUCTURE AND FUNCTION OF THE *TRYPANOSOMA BRUCEI* FLAGELLUM

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Unlike many related parasitic protozoa *Trypanosoma brucei* possesses a flagellum at all stages of its life cycle. In some stages an assessment of the likely function of the flagellum seems to be relatively straightforward. For instance, flagellum mediated motility and subsequent attachment to the epithelium of salivary glands of the insect vector appear central to the completion of the parasite life cycle. After establishment of a mid-gut infection in the tsetse fly, parasites migrate through the gut and peritrophic membrane to complete their differentiation in the salivary glands. Differentiation involves attachment of trypanosomes by their flagellum to host cell surfaces. In the mammalian host, flagellum motility is presumably necessary for the parasite to move from the tsetse bite chancre into the bloodstream and for the establishment of cerebral disease. However, it remains conjectural as to whether there are other reasons for the maintenance of the flagellum in the bloodstream. The basal body that subtends the flagellum does perform an additional and critical role. It is attached to the kinetoplast through a direct connection *via* sets of filaments and a differentiated zone of the mitochondrial membranes: a structure that we have termed the tripartite attachment complex [TAC]. This structure appears to be central to the positioning and segregation of the kinetoplast [1].

At the start of the cell cycle there is one mature basal body which subtends the flagellar axoneme and an immature probasal body. Amongst the earliest detectable morphological events in the cell cycle are the elongation of the existing probasal body and the formation of two new probasal bodies. The three dimensional spatial positioning of these structures changes as the cell cycle progresses.

In addition to the classical "9+2" axoneme, the flagellum of trypanosomes contains a large, unusual lattice-like structure, the paraflagellar rod (PFR). This structure is found exclusively in a restricted set of evolutionary ancient unicellular eukaryotes [2]. The function of the PFR has been the subject of much speculation [3]. In contrast, its molecular constituents are well known. The PFR is composed of two major closely related proteins, termed PFR-A and PFR-C. They share 60 % identity throughout their amino acid sequence but possess different N- and C-

termini. Four copies of the *PFR-A* gene are tightly clustered on each of a diploid chromosome pair and the *PFR-A* mRNA is highly abundant [4].

We have made a new set of monoclonal antibodies that recognise the PFR proteins of *T. brucei*. In addition we have been able to use an epitope tagged version of the *PFR-A* gene, in concert with an inducible gene expression system [5,6], in order to elucidate the mode of incorporation of subunits into the growing PFR structure. The construction of this highly complex structure appears to occur *via* the incorporation of subunits at the distal end. Results from the use of the inducible vector system suggest a linear growth pattern for the structure as a whole. We have also made a deletion mutant analysis of the PFR-A protein which reveals specific portions of the protein that are necesary for translocation and incorporation of the PFR-A subunit into the flagellum.

We have used an antisense approach to ablate PFR-A protein expression in order to study the function of the PFR. Initially, we obtained a particular clone which showed a striking phenotype. The cells grew normally but sedimented to the bottom of the well and appeared paralysed. This mutant was cloned twice by limiting dilution and named *snl*-1. Northern blot analysis showed that only a tiny amount of *PFR-A* mRNA was present in the mutant cell line compared to the wild-type [7].

Western blots on total cell lysates of wild-type and the *snl*-1 mutant were probed with L13D6, a monoclonal antibody recognising both PFR-A and PFR-C from *T. brucei*. Two bands of roughly equal intensity (PFR-A and PFR-C) were detected in the wild-type cells whereas only the PFR-C protein was present in the mutant cell line. Longer exposure of the autoradiogram revealed only a small amount of the PFR-A protein, in agreement with the small amount of *PFR-A* mRNA . This result also implies that the *PFR-C* gene expression was not modified by the antisense produced against *PFR-A*. In wild-type trypanosomes, the PFR proteins were found exclusively in the cytoskeletal fraction and no soluble pool was detected. By contrast, in the *snl*-1 cell line, the PFR-C protein was shifted to the soluble fraction with only a small amount left in the cytoskeletal fraction. Therefore, in the absence of PFR-A protein, the PFR-C protein is still produced but remained soluble, indicating it did not assemble in a cytoskeletal structure.

In immunofluorescence, the monoclonal antibody L13D6, recognising both PFR-A and PFR-C proteins, strongly detects the flagellum of wild-type trypanosomes. A considerably different picture was obtained for the *snl-1* mutant. The immunofluorescence signal is dramatically reduced along the length of the flagellum and in some flagella there was a pronounced dilation of the distal tip. This dilation was strongly stained with the L13D6 antibody, indicating that the non-assembled PFR-C identified above enters the flagellum compartment and accumulates at that site in agreement with our observations with the inducible system. This “blob” of non-assembled material showed cell cycle regulation, suggesting that it was resorbed at some point early in the cell cycle.

Electron microscopic comparison of wild-type and *snl*-1 trypanosomes demonstrated a major ablation of the PFR in the mutant cell line. Cross sections of the wild-type flagellum revealed the typical PFR structure composed of 3 regions defined by their position relative to the axoneme. Connections to the axoneme, always through doublets 4 to 7 and to the flagellum attachment zone are also observed. In the *snl*-1 cells, the intermediate and distal regions of the PFR were completely missing and only a fraction of the smaller proximal region remained. . The connections between this rudimentary PFR structure and the axoneme, as well as those to the flagellum attachment zone were still present.

In the mutant cell line, video microscopy analysis showed that although the polarity was not affected, the frequency and the amplitude of flagellar beating pattern were drastically reduced. Motility is compromised so much in the *snl*-1 cells that they sedimented at the bottom of the flask rather than staying in suspension. Our use of an antisense approach produced a dramatic mutational phenotype in trypanosomes leading to ablation of the PFR and resulting in almost complete paralysis of the cells. This work represents the first major cellular function in trypanosomes revealed by an antisense strategy. This molecular approach also gives insight into the dynamic traffic of proteins in and out of the flagellum compartment. Development of the antisense methodology has allowed us to demonstrate the essential role of the PFR in flagellum and cell motility in trypanosomes. Moreover, these approaches will be useful in studies of flagellum function *in vivo* both in the insect vector and mammalian host.

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RT10 – IMMUNOPATHOLOGY IN LEISHMANIASIS

RT10 - ABILITY OF *LEISHMANIA*-INFECTED MACROPHAGES TO PRESENT THE PARASITE ANTIGEN LACK TO MOUSE CD4$^+$ T CELLS

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The location of *Leishmania* in an endocytic compartment of macrophages (Mf) is in agreement with the preferential triggering of the CD4$^+$ T cell population in infected hosts. However, whether *Leishmania*-infected Mf are antigen-presenting cells for *Leishmania*-specific CD4$^+$ T lymphocytes is still a matter of debate. Results presented here are focused on the ability of *Leishmania*-infected mouse Mf to present the parasite antigen LACK to CD4$^+$ T cell hybridomas. A few hours after infection, promastigote-infected Mf can present LACK but the level of hybridoma stimulation depends on the promastigote stage used for the infection. Indeed, Mf infected with Log-phase or stationary-phase promastigotes are much more efficient to present LACK than Mf infected with metacyclic promastigotes. At later times after infection, promastigote-infected Mf loose their ability to present LACK. On the other hand, whatever the time after infection, amastigote-infected Mf are unable to activate the LACK-reactive T cells. Thus, presentation of LACK seems to correlate with the degree of virulence of the phagocytosed parasites, the less virulent being the best for the generation of LACK epitopes. These differences are not linked to the parasite burden nor to the ability of infected Mf to process LACK as these cells are able to stimulate hybridomas in the presence of a recombinant form of LACK. Semi-quantitative Western blot analysis on parasite lysates and immunostaining on recently phagocytosed parasites show that metacyclics generally express less LACK protein than the other parasite stages. However, in the case of *L. amazonensis* infection for example, Mf infected with metacyclics and containing more parasites (8x) than Mf infected with Log-phase promastigotes are less efficient (factor 6) to stimulate a LACK-specific hybridoma. Thus, activation of LACK-reactive T cells by *Leishmania*-infected Mf is not linked to the level of LACK expression. Morphological studies on Mf indicate that a large part of Log-phase and stationary-phase promastigotes are rapidly destroyed and, very likely release the LACK protein in the phagosome lumen, a compartment where class II and H-2M molecules are detected. This suggests that early phagosomes have already acquired some of the characteristics of the late endocytic compartments and, by inference that the presentation of LACK involves newly-synthesized class II molecules. This assumption is sustained by the fact that the presentation of LACK is blocked by Brefeldin A, a drug which interferes mainly with the classical antigen presentation pathway. Although parasite killing is required for LACK presentation, it is not a sufficient condition as killing of intracellular amastigotes does not allow presentation of LACK by cured Mf. Taken together, these results suggest that virulent stages of *Leishmania* have the capacities to circumvent the antigen presentation process of the host cell.

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RT10 - EFFICACY OF TREATMENT OF CUTANEOUS LEISHMANIASIS WITH INTRALESIONAL HUMAN RECOMBINANT GRANULOCYTE MACROPHAGE COLONY STIMULATING FACTOR (HRGM-CSF) COMBINED WITH ANTIMONIAL

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Pentavalent antimonials are standard treatment of cutaneous leishamniasis (CL). However, there are associated complications, such as: daily iv injections; adverse reactions; delayed treatment response. GM-CSF has the ability *in vitro* to kill *Leishmania* and has been used to treat ulcers of different etiology. The objective of this work was to evaluate the effect of intralesional rGM-CSF as adjuvant therapy in reducing the healing time of ulcer. Twenty CL patients were selected for a double blind randomized trial. Ten patients received two intralesional doses of 200 mg of rGM-CSF at a week interval and antimonial therapy (20mg/Sb^V/kg of body weight per day, during 20 days, iv). The control group received intralesional saline instead of rGM-CSF. The patients were evaluated at a 30 days interval for six months and healing criteria was total scar of the ulcer.

The GM-CSF group had significantly increased cure of the ulcers at 40 days in comparison to the control group (RR 7.00, 95% CI 1.04 to 46.97; $p<0.05$). Although not significant, the GM-CSF group had low cases that require retreatment with antimonial (2/10 vs 4/10). There was no difference in the following parameters: age, size, duration of disease and Montenegro skin test. The mean $\pm$ SD of healing time of the rGM-CSF group 49$\pm$38 days and control group was 110$\pm$62 ($p=0.02$). GM-CSF increased the levels of IFN-g from PBMC cells and also lymphoproliferative

response. This study demonstrates that intralesional rGM-CSF given as adjuvant therapy with antimonial reduces the time for cure from CL ulcer, that can be mediated by immune response and also by the abillity of GM-CSF to induce scar formation.

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RT10 - *IN SITU* INFLAMMATORY-IMMUNE RESPONSE IN HUMAN TEGUMENTARY LEISHMANIASIS: MORPHOLOGIC EVIDENCE FOR A PATHOGENIC ROLE OF TNF-a

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Tegumentary leishmaniasis (TL) represents a major public health problem in South America, including Brazil. A broad spectrum of disease forms are associated with TL and include localized cutaneous (LCL), mucocutaneous leishmaniasis (MCL) and diffuse cutaneous leishmaniasis (DCL)[1]. Expression of disease forms is influenced by host factors such as the immune response. During *Leishmania braziliensis* infection, patients initially present with LCL. An adequate specific cell-mediated immune response is mounted against the parasite. Histologically, few or no parasites are found in the lesions. However, granulomas, vasculitis and foci of fibrinoid necrosis suggest a cell-mediated hypersensitivity mechanism of injury[2]. A subset of patients develop mucosal disease (MCL), involving disfigurement at the nose, pharynx, palate, and upper lip. MCL is characterized by exacerbated cell-mediated immunity and regarded as the hyper-responsive pole of the disease. Parasites are few or absent in the lesions as in LCL but necrosis and tissue destruction are more extensive. The anergic pole associated with diffuse cutaneous leishmaniasis (DCL) is rarely seen. When encountred in Brazil, *L. amazonensis* is the predominant etiological agent.

In the murine model of leishmaniasis, protection is mediated through activation of Th1 cells, which produce IFN-g and IL-2 , whereas susceptibility is related to activation of Th2 cells, which produce IL-4 and IL-10[3]. However, this dichotomy has not been observed in human leishmaniasis using methods such as cytokine mRNA expression in LCL, MCL or DCL lesions[4-6]. Expression of both Th1 and Th2 of cell-mediated immune response has been observed in LCL and MCL indicating that cytokines others than those typically associated to Th1 or Th2 types are involved in human immunopathogenesis. TGF-b has been shown to increase the susceptibility of human macrophages to infection with different species of *Leishmania* and to be expressed in the site of the lesions in early cutaneous leishmaniasis and active MCL. In contrast, TNF-a, plays an important role in protection against *Leishmania* in experimental murine leishmaniasis[8]. High levels of serum TNF-a is not associated with protection or favorable outcome in human infections[9]. Althought high levels of serum TNF-a have been suggested to be involved in pathogenesis, differences in the intralesional TNF-a mRNA expression have not been observed between LCL and MCL. However, several caveats need to be considered when interpreting results from quantitative RT-PCR measurement of cytokine mRNA. First, the detection of mRNA may not correspond to the protein production *in situ.* Second, the types of cells that produce the specific cytokine can not be identified. Third, leishmanial lesions are pleomorphic . Cytokine production in different areas of the lesion may be related to different pathogenetic mechanisms. To adress this problems immunohistochemical methods were applied to investigate the role of cytokines in the pathogenesis of different disease forms of ACL. Biopsies were obtained from 46 patients [LCL (35), MCL (5) DCL (3)]. Immunoperoxidase staining was performed to identify the inflammatory cells phenotypes and to examine the presence and distribution of TNF-a, TGF-b, IFN-g and iNOS. Our results showed that inflammatory infiltrates were composed of macrophages, $CD4^+$ and $CD8^+$ T lymphocytes and plasma cells. Granulocytes were seen in relation to areas of ulceration, vasculitis and necrosis. There was a higher proportion of $CD4^+$ than of $CD8^+$ cells in the lesions. In DCL inflammatory infiltrate was homogeneous and composed almost exclusively by densely parasitized macrophages. Few lymphocytes were present in the periphery of the lesions and showed $CD4^+$ and $CD8^+$ phenotypes. TGF-b positive macrophages and fibroblasts were observed in cases of LCL and MCL. In DCL, TGF-b positive cells were not present. IFN-g positive lymphocytes were observed between cells of the inflammatory infiltrate in cases of LCL and MCL. In only one case of DCL, few lymphocytes were positive. A striking result was observed with respect to the expression of TNF-a. Expression of this cytokine was not observed in the lesions from DCL, except in one patient with sub-polar type where a small isolated granuloma was found. In lesions from patients with LCL and MCL, TNF-a was expressed in isolated mononuclear cells, lymphocytes and macrophages around vessels, macrophages of granulomas, multinucleated giant cells, and surounding areas of necrosis and apoptosis. Expression of TNF-a was associated with the cell membrane, the cytoplasm of inflammatory mononuclear cells or with the extracellular matrix. Membrane-associated TNF-a was frequently observed in mononuclear cells located around vessels. In these cases, vessels showed activated endotelial cells and migration of inflammatory cells through its walls. Vasculitis was observed frequently associated with thrombosis and necrosis of small vessels. In areas of tissue necrosis, large amounts of TNF-a was observed in the extracellular matrix and macrophages. When observed, it was associated primaraly with cases of MCL. Expression of iNOS was observed in

few cells in all forms of ACL. Interestingly, in the cases of DCL, only macrophages that were not parasitized expressed iNOS. These cells could be seen among heavily parasitized macrophages that did not express the enzyme.

In conclusion, results provide morphological evidence for a dual role of TNF-a in tegumentary leishmaniasis. TNF-a appears to play a role in controlling infection and the development of a granulomatous reaction associated with good prognosis. TNF-a activates endothelial cells and promotes migration of the inflammatory cells to the site of the infection. Furthermore, absence of TNF-a expression coincides with heavy parasitism, uncontrolled infection and poor migration of inflammatory cells observed in DCL. However, the soluble form of TNF-a is associated with the necrosis and the extensive tissue destruction observed in MCL and, to a lesser extent in LCL.

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RT10 - THE PATHOGENESIS OF POST KALA AZAR DERMAL LEISHMANIASIS IN SUDAN

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Post kala azar dermal leishmaniasis (PKDL) affects about 50% of the Sudanese patients treated for visceral leishmaniasis (VL) caused by *Leishmania donovani* (Zijlstra et al. 1994). Clinically the dermatosis is characterised by the development of macules, papules or nodules, affecting the face but sometimes also other parts of the body in patients who have been apparently successfully treated for VL (Elhassan et al.1992). In most cases the condition appear in the first months after VL treatment, and most patients spontaneously heal their lesions (Zijlstra et al. 1995, Gasim et al. 1997), however in some patients the disease persists for years despite repeated treatment attempts. Histologically the condition is characterised by inflammation and the presence of scanty number of parasites in the skin (Elhassan et al. 1992). It has been a puzzle why the severe systemic infection characteristic of kala azar is followed by skin symptoms after apparently successful drug treatment, but it was suggested that failure to comply with the extended drug regimen could be a precipitating factor. Initiated by Professor Elhassan, Institute of Endemic Diseases our groups at the Universities at Khartoum and Copenhagen have conducted two longitudinal studies during the last four years which have shed some light on the pathogenesis of PKDL. In the first performed by Soha Gasim, we followed patients from they were diagnosed with kala azar until some of them developed PKDL. In the second performed by Ahmed Ismail we followed patients from they were diagnosed with PKDL until they had healed the lesions following drug treatment.

The main observations were: (a) at diagnosis of VL, parasites were detected in skin biopsies from normal looking skin of all patients. There was little or no skin inflammation at this point, but in all patients who later developed PKDL, keratinocytes produced IL10. There was no production of IL10 by keratinocytes in patients who did not develop PKDL later (Gasim et al. 1998). (b) At diagnosis of PKDL, parasites could also be detected in skin biopsies from lesions. However, at this time point there had been a considerable influx of inflammatory cell, most of which were CD4 positive (Ismail et al. 1997). (c) At diagnosis of VL, PBMC did not respond to *Leishmania* antigens *in vitro*. After treatment of VL the responsiveness increased and PBMC from patients proliferated and produced IFNc in response to *Leishmania*. Development of PKDL was associated with an increased ability of PBMC to respond to *Leishmania* antigens in vitro, and there was little difference in the responsiveness between those who developed PKDL and those who did not develop PKDL.

The conclusions from these studies are that parasites spread to the skin during VL, at this time the immune response is biased towards Th2 type responsiveness (reviewed by Kemp et al. 1997), and the parasites in the skin do not cause inflammation. In some VL patients, the parasites induce IL10 production by keratinocytes and this allows the parasites in the skin to survive the drug treatment. During drug treatment of VL, the immune response to

Leishmania is gradually moving from the Th2 type response seen in VL to the mixed Th1/Th2 type response seen when patients have been treated for VL. As the Th1 type responsiveness picks up, an immunological attack is launched against the parasites, which in some patients have survived in the skin, and PKDL develops.

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RT11- ENTAMOEBA

RT11 - ECTO-ENZYMES PRESENT IN *ENTAMOEBA HISTOLYTICA*

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Amebiasis by the protozoan *Entamoeba histolytica* is one of the leading causes of morbidity and mortality in the world. It has been estimated that 10% of the global population is infected , it is the third cause of death among parasitic diseases, only after malaria and schistosomiasis. The enteric protozoan parasite *Entamoeba histolytica* is one of the most potent cytotoxic cells known (Ravdin et al. 1989 *Rev Infect Dis 4*: 1185-1207). Several theories have been advanced regarding specific characteristic of the parasite and potential mechanisms that may be responsible for the remarkable tissue-destroying "virulence". Surface membrane interactions between *E. histolytica* and its hosts are of critical importance and an understanding of the biochemical basis for the parasite adherence and cytolytic activity mechanisms are prerequisites for vaccine development (Radvin 1995 *J Infect Dis 20*: 1453-1466). It has been described that the plasma membrane of cells may contain enzymes whose active sites face external medium rather than the cytoplasm. The activities of these enzymes, referred to as ecto-enzymes, as well as those of some secreted enzymes can be measured using live cells. Of particular interest are the ecto-enzymes present in protozoan parasite, since they are in close contact with the host cells (Furuya et al. 1998 *Mol Biochem Parasitol 92*: 339-348). We have recently characterized two ecto-enzymes in parasitic protozoa of family Trypanosomatidae, an ecto-ATPase present in *Leishmania tropica* (Meyer-Fernandes et al. 1997 *Arch Biochem Biophys 341*: 40-46) and an ecto-protein tyrosine phosphatase present in *Trypanosoma cruzi* (Furuya et al. 1998 *loc. cit.*). Here we show that living *E. histolytica* HM1:IMSS strain is able to hydrolyze extracellular *p*-nitrophenylphosphate (*p*-NPP) and ATP. The parasites were grown in TYI-S-33 medium for 24-48 hr at 37°C. Cellular viability was assessed, before and after incubations, by use of eosin methods. The ecto-phosphatase and ecto-ATPase activities were linear with time at least for one hour. It was also observed that increasing the cell density results in a proportional increase in these activities. We observed that living cells of *E. histolytica* present a low level of ATP hydrolysis in the absence of divalent metal (74 nmoles Pi/h/10^5 cells). However in the presence of 5 mM $MgCl_2$ the ecto-ATPase activity was 280 nmoles Pi/h/10^5 cells. The ATPase described here is also stimulated by $MnCl_2$ and $CaCl_2$ but not by $SrCl_2$, $ZnCl_2$ or $FeCl_2$. In the pH range from 6.4 to 8.4, in which the cells were viable, the ecto-phosphatase activity decreased, while the Mg-dependent ecto-ATPase activity increased, and the Mg-independent ATPase activity did not change. To confirm that the observed ATP hydrolysis was neither attributed to phosphatase nor 5'nucleotidase activities, a few inhibitors for these enzymes were tested. Sodium vanadate, ammonium molybdate and sodium fluoride (NaF) strongly inhibited the phosphatase activity; however, no effect was observed on ATPase activity. Regarding the effects of vanadate and fluoride on phosphatase activity, it must be stressed that micromolar concentrations of vanadate, described as selective phosphotyrosyl phosphatase inhibitor, could be indicating that this ecto-phosphatase activity may also be able to catalyze dephosphorylation of tyrosine phosphorylated proteins. Levamizole a specific inhibitor of alkaline phosphatases and sodium tartrate (inhibitor of secreted phosphatase) failed to inhibit these activities. The lack of response to ammonium molybdate also indicated that 5'nucleotidase did not contribute to the ATP hydrolysis. Other nucleotides such as CTP, GTP, ITP, UTP, and ADP produced lower reaction rates. The

inhibition of the g^{32}ATP hydrolysis by ADP suggests that the ATP hydrolysis may be promoted by any ATP diphosphohydrolase activity. The physiological role of ecto-ATPases is still unknown, however, several hypothesis have been suggested, such as protection from cytolytic effects of extracellular ATP and involvement in cellular adhesion. The virulent *E. histolytica* presents high level of ATP hydrolysis (74 nmoles Pi/h/10^5 cells in the absence of $MgCl_2$ and 280 nmoles Pi/h/10^5 cells in the presence of $MgCl_2$). The non-virulent *E. dispar* presents a lower level of ATP hydrolysis (33 nmoles Pi/h/10^5 cells in the absence of $MgCl_2$ and 90 nmoles Pi/h/10^5 cells in the presence of $MgCl_2$). The physiological role of these ecto-ATPase activities in *E. histolytica* and the possibility of these enzymes to be markers of virulence remains to be elucidated.

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RT11 - MOLECULAR SYSTEMATICS OF PARASITIC AMEBAE

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Four genera of amebae live in the colon of humans of which *Entamoeba* is the most widely studied. A simple life cycle consisting of an infective cyst stage and a multiplying trophozoite stage is found in the genera *Endolimax* and *Iodamoeba* and most species of *Entamoeba*, but the fourth genus, *Dientamoeba*, has no known cyst stage and its transmission is not understood. Three of the genera are represented by a single species in humans, but as many as eight species of *Entamoeba* have been reported with varying frequency. However, because species identification is not straightforward not all these reports are accepted - as is the case with most amebae there are few reliable morphological criteria available. Molecular tools can be used to provide additional characteristics for the recognition of established and new species. In this presentation, our current understanding of variation within and between species will be examined as well as what we know of the relationships between these parasites and other eukaryotes. We have used small subunit ribosomal RNA genes (rDNA) to address each of these three areas.

Relationships within genera - All the morphologically defined species of *Entamoeba* were supported by riboprinting - restriction fragment length polymorphism analysis of polymerase chain reaction amplified small subunit rDNA. However, in three species intraspecific variation was detected and in two cases, *E. coli* and *E. moshkovskii*, the amount of variation was greater than between several other pairs of species. We use the term 'ribodeme' to recognize these variants since the riboprint patterns are the only information we have about most of these organisms and we do not feel that the description of new species is justified at this time.

A number of additional observations can be made from the riboprint data. *Entamoeba* species have classically been grouped based on the number of nuclei seen in mature cysts, and these groupings are also found by riboprinting. *Entamoeba gingivalis* does not encyst, but it clearly clusters with the species producing cysts with four nuclei. Within the group producing cysts with four nuclei, species derived from mammalian sources are interspersed with those from reptiles.

Although only a few isolates have been examined, riboprinting of *Dientamoeba fragilis* has revealed the existence of two ribodemes so far. Whether this diversity is linked to the uncertainty over the organism's role in disease remains to be determined. Riboprints of two *Endolimax nana* isolates were identical. Unfortunately, we have so far been unsuccessful in obtaining material for analysis of *I. bütschlii*.

Relationships among genera - Phylogenetic analysis of the *D. fragilis* small subunit rDNA sequence confirmed that it is a trichomonad, as had been predicted by electron microscopy. It appears to diverge early in the trichomonad radiation but this is not well resolved. It is now clear that *D. fragilis* has secondarily lost the characteristics of a 'typical' trichomonad, rather than being a primitive form - the recent identification of earlier branching organisms in the trichomonad lineage as members of the genus *Trichonympha* confirms this view.

Because of the large distances detected between species of *Entamoeba*, the small subunit rDNAs of many of the species were cloned and sequenced. Phylogenetic analysis resulted in trees that supported the conclusions based on riboprinting but gave more resolution.

The order of divergence among the three major lineages of *Entamoeba* could not be determined even when the *Entamoeba* tree was rooted - the ancestral number of nuclei in a cyst could not be resolved, therefore. The position of *Endolimax nana* in the eukaryotic tree is very close to the base of the *Entamoeba* branch and in some analyses *Endolimax* and *Entamoeba* form a clade. It is possible that the classical view of these two genera belonging to the same family - Entamoebidae - will ultimately prove to be correct, but it is clear that they are not specifically related to *Dientamoeba* as had been originally thought.

Relationships with other eukaryotes - We cannot be sure that characteristics found in both *Entamoeba* and *Endolimax* are the result of common ancestry rather than convergence. Amebae are polyphyletic based on the rDNA sequence data available so far. The ameboid cell form appears to be a 'default' state for eukaryotic organisms and has arisen a number of times in unrelated lineages. Secondary loss of the cytoskeleton and secondary loss of organelles both appear to be quite frequent events in eukaryotic evolution. The absence of either character should not be taken as evidence for a primitive state.

In the global tree of eukaryotes, the positions of *Endolimax* and *Entamoeba* indicate that both are descended from an ancestor that had mitochondria and other typical eukaryotic organelles. However, recent discoveries of

genes of mitochondrial origin in every major groups indicates that perhaps all known eukaryotes are descended from an ancestor that had these characteristics. While the hydrogenosome of *Dientamoeba* and other trichomonads provides a clear link to the mitochondrion, the presence of an organelle of mitochondrial origin in *Entamoeba* has not been suggested by electron microscopy. Our recent results show that at least one of the proteins of mitochondrial origin detected in *E. histolytica* is located in a sedimentable compartment. This may be a remnant of the mitochondrion, although what its function is in *Entamoeba* remains to be determined.

RT12 – PARASITE POPULATION AND MOLECULAR BIOLOGY

RT12 – EXPRESSION, PROCESSING AND CELLULAR TRAFFICKING OF A *TRYPANOSOMA CRUZI* SURFACE GLYCOPROTEIN (GP82) IN A MAMMALIAN CELL BACKGROUND

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Metacyclic trypomastigotes of *Trypanosoma cruzi* express a developmentally regulated 82 kDa surface lycoprotein which has been implicated in the invasion of the mammalian host cells by the parasite. Analysis of cDNA clones encoding gp82 revealed the existence of several post-translational modifications such as *N*-glycosylation and addition of a GPI anchor. Although many features of the biosynthesis of surface membrane proteins have been conserved in the evolution, the requirements for translocation of the nascent peptide chain across the reticulum endoplasmic membrane and lycosylphosphatidylinositol (GPI) anchor attachment may be not the same in mammals and parasitic protozoa. The goal of present work was to test whether the gp82 signals function in mammalian cells.

We have analyzed the expression of gp82 protein in Vero and Cos-7 cells transfected with the plasmid pcDNA3 carrying the complete open reading frame of gene *gp82*. The gp82 cDNA was subcloned in plasmid pcDNA3 (construct pcDNA3-gp82) maintaining the putative parasitic sequences for addition of GPI anchor and the signal peptide (NH_2-terminal cleavable sequence). Transfected cells expressed four unsoluble polypeptides under 60 kDa suggesting that the signal peptide found in gp82 was not recognized by the mammalian cell system responsible for the sorting of membrane proteins.

To test this hypothesis, the signal peptide sequence from the haemagglutinin virus protein was cloned in frame with the gene *gp82* generating a new construct named pcDNA3-gp82+SP. We have compared the expression of both pcDNA3-gp82 and pcDNA3-gp82+SP by immunoblotting, immunofluorescence and *N*-glycosylation inhibition through tunicamycin. Transfected cells with pcDNA3-gp82+SP expressed a 75 kDa protein detected with Mab3F6 (specific for gp82). When the transfection was made in presence of tunicamycin a faster migrating form of gp82 (70 kDa) was detected. It had a mobility on gel matching that of deglycosylated gp82. Transfected cells with the construct pcDNA3-gp82 expressed the same four polypeptides (under 60 kDa) in the presence or absence of tunicamycin.

A further indication that the post-translational processing of gp82 was blocked in Vero cells was obtained from the analysis of processing of gp82 in canine pancreatic microsomal membranes. Translation reactions were directed by mRNA templates transcribed in vitro from pcDNA3-gp82+SP and pcDNA3-gp82 constructs. RNA transcripts were translated in rabbit reticulocyte lysate in the presence of ^{35}S-methionine and processing events were examined by adding canine microsomal membranes to the standard translation reaction. Translation of transcripts from pcDNA3-gp82-SP in rabbit reticulocyte lysate produced a polypeptide of about 56 kDa. After addition of microsomal vesicles in the system, a broad upper band of ~75 kDa appeared suggesting that the 56-kDa precursor was translocated into vesicles and processed. Two polypeptides of 56 kDa and 45 kDa were translated from pcDNA3-gp82 transcripts and this pattern was not altered by adding microsomal vesicles.

To evaluate whether the *N*-linked oligosaccharides on gp82 are of high mannose or complex type, native and recombinant gp82 proteins were treated either PNGaseF, an enzyme with broad specificity for *N*-linked oligosaccharides, or endo H, an enzyme with specificity for high mannose oligosaccharides. Treatment of native and recombinant gp82 with PNGaseF or endo H generated a lower molecular mass polypeptide that comigrated with the precursor of gp82 identified in Vero cells after treatment with tunicamycin. The sensitivity of gp82 to endo H digestion and inhibition of its glycosylation by tunicamycin indicate that the addition of high mannose oligosaccharide chains follows the general pathway for *N*-linked oligosaccharides. Acquisition of endo H resistant complex carbohydrate has been used to monitor the movement of an intracellular protein from the endoplasmic reticulum to the medial Golgi compartment. Immunofluorescent staining of transfected Vero cells suggested that part of gp82 is transported out of the endoplasmic reticulum and accumulates in a cellular compartment that colocalizes with wheat germ agglutinin, a known Golgi marker.

These results suggest that the peptide signal from haemagglutinin protein gene in frame with gp82 gene allowed endoplasmic membrane targeting ad glycosylation of gp82 protein in transfected cells. Most likely indicating that the signal sequence processing in higher eucaryotic cells is distinct from that of trypanosomatids. These differences could be useful for the development of specific antiparasite drugs.

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RT12 - EVOLUTION AND MOLECULAR EPIDEMIOLOGY OF NEW WORLD *LEISHMANIA*

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Numerical analyses, using phenetic and phylogenetic methods, have questioned the proposed classification of *Leishmania* in two subgenera by Lainson and Shaw (1987 *The Leishmaniasis*, vol. 1, p.1-20). The *Viannia* subgenus is autochthonous to the New World and represents one cluster, suggesting a monophyletic group. However, the *Leishmania* subgenus is represented in the Americas by the etiological agents of visceral leishmaniasis, *L. (L.) chagasi*, and several other groups of human and non-human *Leishmania* pathogens, suggesting the classification of these parasites in more than one cluster (Cupolillo et al. 1994 *Am J Trop Med Hyg 50*: 296). Using molecular and biochemical methods we have demonstrated that some New World *Leishmania* species, from both *Viannia* and *Leishmania* subgenera (including human species) are close related to *Endotrypanum* (parasite that cause infection exclusively in sloths), reinforcing the idea of a neotropical origin of *Leishmania* (Noyes et al. 1997 *J Euk Microbiol 44:* 511).

Our results have shown great genetic diversity among New World *Leishmania* parasites. Biochemical and molecular characterization of species within the genus has revealed that much of the population heterogeneity has a genetic basis, which appears to arise from predominantly asexual, clonal reproduction (constant mutation), although occasional bouts of sexual reproduction (genetic recombination) can not be ruled out. Genetic variation is extensive with some clones widely distributed, while others are seemingly unique and localized to a particular endemic focus (Cupolillo et al. 1997 *Ann Trop Med Hyg 91*: 617). Knowledge of the selection process generating such diversity and the epidemiological significance of such variation not only has important implication for the control of the leishmaniases, but would also help to elucidate the evolutionary biology of the causative agents.

Epidemiological studies of Leishmaniasis has been directed to the ecology and dynamics of transmission of *Leishmania* species/variants, particularly in localized areas. Characterization by enzyme electrophoresis and RFLP of the ITSrRNA of *L. braziliensis* from the Amazonian basin have indicated a high level of genetic variation and *L. naiffi* showed intraspecific distances comparable to the largest ones obtained among other *Viannia* species. However, *L. braziliensis* populations from two endemic areas in the Brazilian southeast coast showed low levels of heterogeneity. Interesting, these results are apparently related with the level of diversity of vertebrate host and phlebotomine sandflies involved in the transmission cycles of the parasites. The epidemiological significance of such variation has important implications for the prevention and control of the Leishmaniases.

RT12 - THE ANCIENT AND DIVERGENT ORIGINS OF SLEEPING SICKNESS AND CHAGAS DISEASE, WITH SPECIAL REFERENCE TO THE MAN INFECTIVE TRYPANOSOMES OF LATIN AMERICA

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This paper presents important new findings concerning the evolution of the human pathogens, *Trypanosoma cruzi* and *T. brucei*, which suggest that these parasites have divergent origins and fundamentally different patterns of evolution. Phylogenetic analysis of 18S rRNA sequences and preliminary minexon data places *T. brucei* in a clade comprising exclusively mammalian trypanosomes of African origin, suggesting an evolutionary history confined to Africa; *T. cruzi* and *T. rangeli* sequences cluster with a range of *T.* (*Schizotrypanum*) trypanosomes in a separate clade, the origins of which lie in South America and Australia.

Viewed in the context of palaeogeographic and fossil evidence, the results date the divergence of *T. brucei* and *T. cruzi*/*T. rangeli* to the mid-Cretaceous, suggesting an ancient southern super-continent origin for *T. cruzi* and related species, possibly in marsupials and long before the emergence of their present day triatomine vectors. Thus, while *T. brucei* appears to have co-evolved with humans and early hominids for more than 8 million years, *T. cruzi* has effectively evolved in the absence of man, becoming a human pathogen only in the last few thousand years.

RT13 – APICOMPLEXA

RT13 – IDENTIFICATION AND CLONING OF A PARAFUSIN HOMOLOGUE IN *TOXOPLASMA GONDII*

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Toxoplasma gondii, an obligate intracellular protozoan parasite, contains 3 types of secretory organelles that it secretes upon invasion of host cells. The regulation of this process is not known. We have used earlier studies from the ciliated protozoan, *Paramecium tetraurelia*, as a paradigm for exocytosis in *T. gondii*. In *Paramecium*, parafusin (PFUS), a member of the phosphoglucomutase superfamily, plays a role in regulated secretion and may be involved in the Ca2+-dependent signal transduction event(s) that regulate membrane fusion in exocytosis. PFUS in this cell associates with the secretory vesicles and with the cell membrane docking sites prior to exocytosis and it dissociates from these sites after secretion. The EST database for *Toxoplasma* revealed four clones with homology to PFUS. These have been amplified and used to screen an RH cDNA library. A PFUS homologue in *T. gondii* - parafusin-related protein (PRP1) - has been cloned and sequenced. This protein has been expressed in *Escherichia coli*. The calculated molecular weight of the clone is 70,000 D. Western blot analysis of *T. gondii* lysates probed with a specific I-2 peptide antibody raised to *Paramecium* PFUS confirms the presence of a PFUS homologue at Mr 68,000 in *T. gondii*. The expressed recombinant PRP1 is also recognized in Western blots by the I-2 antibody. Immunofluorescence microscopy of tachyzoites using the I-2 antibody showed localization that corresponds to the positions and shapes of the secretory organelles. Double labeling experiments using the I-2.

RT13 - REDISTRIBUTION OF PARASITE AND HOST CELL MEMBRANE COMPONENTS DURING *TOXOPLASMA GONDII* INVASION

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When tachyzoites of *Toxoplasma gondii* enter in contact with the surface of host cells induce a special endocytic process which leds to the formation of a vacuole known as the parasitophorous vacuole (PV). It has been suggested that during formation of the PV host cell plasma membrane components are excluded from the forming parasitophorous vacuole membrane (PVM) avoiding later fusion with the host cell endocytic system (7,17). The origin of the PVM in cells infected with *T. gondii* and other Apicomplexan parasites is not completely clarified and has been subject of considerable controversy (23,10). Two main models have been proposed to explain how the PVM is formed: (a) in the bilayer insertion model, the PVM is thought to be formed from lipids that the parasite secretes from apical organelles and insert into the host cell membrane during invasion; (b) an alternative model, known as induced invagination, proposes that the parasite induces the host cell membrane to invaginate to form the PVM (20).

The composition of lipids and proteins in the parasitophorous vacuole is unknown, but lipids were likely to influence the ability of this compartment to interact with other vesicles within the host cell (17). The phospholipids in the parasitophorous vacuole membrane surrounding *Plasmodium knowlesi* are primarily derived from invagination of the red cell plasma membrane (23). A similar process in involved in the formation of parasitophorous vacuole with *T. gondii*. Electron microscopy studies indicated that the vacuole is formed by invagination of the host cell plasma membrane as soon as the parasite invade (12).

During *T. gondii* invasion process, some host cell plasma membrane proteins are present in newly formed parasitophorous vacuoles, but are rapidly removed and are no more observed in mature vacuoles, and other membrane proteins are excluded during the formation of the vacuole (23,7). Immunoelectron microscopy and freeze-fracture studies have shown that erythrocyte membrane proteins are essentially absent from the PVM in *P. knowlesi* (2,4) and significantly reduced in PV containing *T. gondii* (14).

Assuming that during the process of *T. gondii*-host cell interaction interchange of surface components of the two cells takes place we analyzed the parasite-host cell interaction process using either parasites or host cells whose membrane was previously labeled with probes specific for proteins, sialoglycoconjugates and lipids and then allowed to interact for periods varying from 5 min to 24 hr. The fate of the fluorescents probes was followed by confocal laser scanning microscopy. In host cells previously labeled with PKH26, FITC-Thiosemicarbazide or DTAF, which label membrane proteins, siloglycoconjugates and lipids, respectively, a uniform labeling of the cell surface was observed before interaction.

The fluorescent lipophilic probe PKH26, that binds irreversibly within cell membranes (9), was used to label the host-cell membrane and to follow the fate of lipids during PV formation in cells allowed to interact with *T. gondii*. Our present observations show clearly that during the internalization process labeled portions of the host cell plasma membrane are internalized and will be part of the PVM. In addition, later on the intravacuolar parasites

release proteins located within the dense granules, such as GRA1, GRA2 and GRA4, which remain in the vacuole (7) and are also incorporated into the intravacuolar membranous network and the PVM (23). With the evolution of the intravacuolar parasitism the intensity of labeling decreases, possibly due to dilution of the label among the new parasites formed after division (3). Observation of malaria invasion in red blood cells labeled with fluorescent lipids clearly showed that the vacuole originated by invagination of the red cell membrane (23). Joiner (10) suggested the insertion of lipids of the parasite on PVM, that might affect its interaction with other cellular membranes preventing fusion with endocytic compartments. Suss-Toby (22) reported that a small amount of parasite-derived material (0-18.5% of the total surface area of the PVM) may be inserted into the host cell plasma membrane. Our present observations can not definitively rule out bulk insertion of lipids as another possible mechanism of PVM formation as previously suggested (10).

We used DTAF, which reacts with primary and secondary amine groups (22), to label membrane proteins of the Vero cells before interaction with tachyzoites in order to follow the fate of the labeled proteins during the parasite-host cell interaction process. As expected, only the Vero cells surface was labeled when incubation was carried out at 4ºC. However, when these labeled cells were incubated in the presence of parasites at 37ºC labeling of the surface of attached parasites was evident. This is an interesting observation which suggests a process of transference of components of the host cell surface to the tachyzoites surface during the early steps of the interaction process. Previous studies have shown that following parasite attachment secretion of microneme components followed by secretion of rhoptry components takes place (11,19,7) and it has been suggested that these secretory molecules play some role on the process of invasion of the host cell by the protozoan. Our present observations suggest that components of the host cell may also be transferred to the parasite, indicating the complexity of the interaction process. Once the parasites were internalized labeling of the PVM and of the intravacuolar parasites was evident, indicating that at least some plasma membrane components of the host cell are internalized and will make part of the PVM. Previous studies using freeze-fracture have shown that the PVM of *T. gondii* - containing PV presents some intramembranous proteins (19). These observations are also in agreement with studies from Carvalho and De Souza (7) and Sibley (17) where in a variety of host-cell types, including macrophages, fibroblasts and epithelial, the cell surface Na/K^+ ATPase was internalized with invading *T. gondii* cells.This pump is only observed in the vacuole during the first few minutes after formation and is absent from mature vacuoles.

Our observations using host cell previously labeled with Fluorescein-thiosemicarbazide showed intense labeling of the surface of parasites attached to the cell surface, suggesting transference of labeled molecules from the host cell to the parasite surface. However, in contrast to what was observed with the labels for proteins and lipids, no labeling of the PVM and intravacuolar parasites was observed. This observation suggests that sialoglycoconjugates exposed on the host cell surface are not internalized during the process of internalization of tachyzoites of *T. gondii*, being excluded in a not yet defined step of the process. This result was unexpected in view of previous studies showing that surface anionic sites, detected using cationizad ferritin particles, were internalized together with untreated tachyzoites and excluded when antibody-coated parasites were used (8). The observation that attached, but not internalized parasites were labeled also suggests removal of surface components of the parasite during the internalization process. However, labeled cytoplasmic structures were seen in the host cell. They probably correspond to organelles of the endocytic pathway formed by invagination of the other regions of the host cell surface not involved in interaction with parasites.

We observed that incubation of tachyzoites in the presence of PKH26, DTAF, and in a less extent, FITC-thiosemicarbazide, labeled their surface. When labeled parasites were allowed to interact with host cells intense labeling of the areas of contact between the parasite and the host cell was observed. However, neither the PVM nor the intravacuolar parasites were labeled, indicating that the surface molecules were released into the extracellular medium before the interiorization process. Previous studies have shown that malaria merozoites labeled with fluorescent fatty acids transfer fluorescent material to the parasitophorous vacuole during invasion (13). Specific proteins (5,16) and lipids (16) are thought to be transferred from the apical organelles during invagination contributing to the formation of the PVM.

Taken together the available data suggest that following the initial contact of tachyzoites of *T. gondii* with the surface of the host cell interchange of surface components of the two interaction cells takes place. This phenomenon, in association with the release of macromolecules found in the micronemes and rhoptries and the invagination of the host cell plasma membrane originates the initial parasitophorous vacuole membrane which surround the parasite-containing vacuole.

These observations point to the existence of a dynamic process of membrane-associated components of the parasite and host cell during the interaction process.

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RT13 - TNF-α, NITRIC OXIDE, AND IFN-γ ARE ALL CRITICAL FOR DEVELOPMENT OF NECROSIS IN THE SMALL INTESTINE OF GENETICALLY SUSCEPTIBLE MICE INFECTED PERORALLY WITH *TOXOPLASMA GONDII*

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We have previously reported that genetic susceptibility (mortality) of C57BL/6 mice to peroral infection with *Toxoplasma gondii*, a protozoan parasite, is associated with necrosis of the villi and mucosal cells in their small intestines and that IFN-γ mediate development of this necrosis. In the present study, we examined whether TNF-α and nitric oxide (NO), in addition to IFN-γ, play an important role in development of the necrosis of the small intestines we observed in infected C57BL/6 mice. At 7 days after infection (when mice had developed necrosis), markedly and significantly greater amounts of mRNA for IFN-γ, TNF-α, and inducible NO synthase (iNOS) were detected in lamina propria mononuclear cells of the small intestines of infected than of uninfected mice. Treatment of infected mice with anti-TNF-α mAb or the iNOS inhibitor, aminoguanidine, prevented development of necrosis as efficiently as did treatment with anti-IFN-γ mAb. Infected iNOS-targeted mutant mice did not develop the necrosis whereas infected control mice did. Furthermore, treatment with anti-TNF-α mAb or aminoguanidine significantly prolonged time to death of infected mice. These results indicate that TNF-α and NO, in addition to IFN-γ, play a critical role in development of necrosis in the small intestines and contribute to early death in genetically susceptible mice following peroral infection with *T. gondii*.

RT14 - ACHIEVEMENTS AND PERSPECTIVES OF CONTROL AND RESEARCH SUPPORT FOR PROTOZOAN DISEASES

Abstracts not received.

RT15 – MOLECULAR BIOLOGY OF TRYPANOSOMATIDS

RT15 - THE NUCLEAR REORGANIZATION CORRELATES WITH THE DIFFERENTIAL TRANSCRIPTIONAL RATES DURING THE DIFFERENTIATION OF THE PROTOZOAN *TRYPANOSOMA CRUZI*

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In trypanosomes transcription occurs in large polycystronic units. Trans-splicing and polyadenylation generate each individual mRNA. There is no defined RNA polymerase II promoters and mRNA stabilization controls the level of differentially expressed mRNAs (1,3,4).

We found a large decrease in the transcription rate when *Trypanosoma cruzi* proliferative forms (epimastigotes and amastigotes) transforms into non-proliferative/infective forms (trypomastigotes). Epimastigotes incorporate about ten times more [^{3}H]-uridine than trypomastigotes. This incorporation is due to RNA polymerase I activity, since it was not inhibited by a-amanitin. RNA polymerase II transcription also decreased in trypomastigotes. [^{32}P]UTP incorporation in nascent mRNAs by lysolecithin permeabilized *T. cruzi*, or by isolated nuclei, was less than ten folds in trypomastigotes. These changes in the transcriptional rate correlates with the nuclear reorganization that occurs when proliferative transforms into infective forms. Trypomastigote nuclei is more susceptible to *Micrococcus* nuclease digestion, suggesting that the transcriptional and processing machinery uncover the nucleosomal structures in the epimastigote nuclei.

The nucleus of proliferative forms is round, contains a large nucleolus, and small amounts of peripheral heterochromatin. The nucleus of trypomastigotes is elongated, the nucleolus disappears, and the heterochromatin occupies most of the nuclear space. In situ hybridization with a probe to satelite sequences (2) typically located in heterochromatic regions shows a peripheral localization in epimastigotes versus a diffuse localization in trypomastigotes.

The nucleolar structure disassemble when the parasite transforms into trypomastigotes as seen by using a GFP-tagged to the nucleolus, or by using a nucleolar-specific monoclonal antibody, suggesting that nucleolus reorganization occurs simultaneously to the decrease in RNA polymerase I transcriptional activity. On the other hand, the increase in the heterochromatin might be related to the decrease in RNA polymerase II transcription through chromatin reorganization. This reorganization was evidenced by differential staining of parasites with monoclonal anti-DNA and anti-chromatin antibodies. The antibodies reacted with proliferative forms but failed to react with formaldehyde-fixed trypomastigote forms. Trypomastigote staining was only revealed by pre-treatment with detergents, or in cross-sections, indicating that the failure to recognize trypomastigote nuclei is due to antibody inaccessibility. We conclude that the changes in morphology and nuclear organization are important to control gene expression in *T. cruzi*.

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RT15 - A STAGE-SPECIFIC ADP-RIBOSYLATION FACTOR-LIKE FROM *LEISHMANIA*

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ARLs (ADP-Ribosylation Factor-like) are small GTP-binding proteins of the ras-superfamily; they are closely related to ARFs (ADP-Ribosylation Factors). While some ARFs have been shown to be essential elements in the vesicle formation and intracellular trafficking, role and localization of ARLs remain elusive. We report here the first characterization and intracellular localization of *ld*ARL-3A, a homolog of human ARL-3, from *Leishmania donovani*. A cDNA and the corresponding unique gene were cloned and sequenced; homolog genes from several other species of *Leishmania* appear also to be unique. Northern blot analyses with mRNAs *L. amazonensis* showed that *ld*ARL-3A mRNA is developmentally regulated; it is much more abundant in promastigotes as compared to amastigotes. Sequence of the predicted protein shows consensus motives of GTP-binding proteins as well as other essential conserved amino-acids. The complete open reading frame (ORF) was cloned in a bacterial expression vector and the protein product purified to almost homogeneity. Rabbit polyclonal antibodies raised against recombinant *ld*ARL-3A, although they might also recognize other ARLs, revealed a single 21-23 kDa band in *L. donovani* and *L. amazonensis* extracts; this band was much more intense in promastigotes than amastigotes. The complete ORF was cloned in pTEX expression vector and stable transformants of *L. amazonensis* were obtained, which overexpressed *ld*ARL-3A; using normal and transformed lines, and polyclonal antiserum, indirect immunofluorescence microscopy showed labelling of discrete elements within the cytoplasm, including the flagellum; patterns appeared similar for both kinds of lines, except labelling was much more intense in transformed lines. Mutant proteins were expressed in *L. amazonensis*, which are deficient in GTP hydrolysis, in GDP to GTP exchange or unable to become myristoylated; phenotypes of the clones are currently under examination. Electron microscopy experiments are also in progress. These new data may give hints for defining the function of *ld*ARL-3A.

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RT15 - PHYSICAL MAPPING OF *LEISHMANIA MAJOR*: A TOOL AND SOME FINDINGS

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One of the major achievements of Genome Projects is the generation of tools for further studies to understand organization and dynamics of genomes, localizing not only genes but also structural sequences such as telomeres and centromeres.

Physical mapping consists of ordering cloned genomic DNA in their original organization onto chromosomes. It can be pursued with different strategies and represents an important step in genome research. It is possible to generate a physical map using a chromosome specific or a random mapping strategy.

The chromosome specific strategy has been carried out combining chromosome walking and non-random STS-content mapping procedures. The walking procedure is time consuming and inefficient to generate a map of the genome as whole, but originates fine maps and constitutes ready-to-use data for the study of many aspects of chromosome structure and genomic organization, presence and distribution of reiterate sequences.

We have used ESTs (*e*xpressed *s*equence *t*ags) to map genes onto genomic clones as an approach to link physical and genetic mapping information and to generate a low resolution physical map randomly across the genome. These ESTs are used in hybridization experiments with the *Leishmania major* - LV 39 (LV39, Rho/Su/59/P) arrayed genomic library. Besides identification of positive recombinant clones, each EST is hybridized to the chromosomes of *Leishmania* separated by pulsed field gel electrophoresis (PFGE). Expressed tags and recombinants are thus grouped according to chromosomal bands. A number of contigs have been produced for the LV 39 genome, the contigs and some analyses of the process will be discussed.

The combination of both chromosome specific and global approaches allowed the confirmation and improvement of contigs generated for the mini-exon chromosome. The chromosome 2 map is complete and includes the telomeric end. This map contains a clone carrying only the miniexon array, which was transfected into an avirulent line of *L. major* to test wether the number of copies of the array would alter the pattern of virulence as suggested previously by Samaras and Spithill (UCLA Symposia on Molec. Cell. Biol., vol 42, p. 269-278, 1986). Only one transfectant (D2 clone, among four) showed significant increase in virulence *in vivo* when they were injected on susceptible animals (BALB/c). To test whether this effect was related to the miniexon extracopies, transfectants were cured. *In vivo* infection experiments have shown that the virulence observed in D2 clone is not related to the increased number of miniexon array. When D2 clone was kept under increasing drug concentration (40 and 80 :g/ml of hygromicin B) we observed a significant decrease in virulence.

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RT16 – DRUG RESISTANCE IN PROTOZOA

RT16 - *IN VIVO* TRIAL FOR EVALUATION OF CHLOROQUINE SENSITIVITY OF *PLASMODIUM VIVAX* ISOLATES FROM RONDÔNIA (AMAZON REGION, BRAZIL) AND EFFICACY OF A NEW THERAPEUTIC SCHEDULES OF ASSOCIATION WITH PRIMAQUINE FOR TREATMENT OF *P. VIVAX* MALARIA

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A therapeutic clinical trial was carried out in patients suffering from *Plasmodium vivax* infections in the State of Rondônia (Amazon Region, Brazil) comparing two schedules of treatment, using double blind methods for selection and follow up of patients: (a) standard doses of Chloroquine (1,5 g in three days) followed by Primaquine (15 mg/day for 14 days) and (b) Chloroquine (1,5 mg in 5 days) administered simultaneously with Primaquine (150 mg/day for 5 days).

The drugs used in the trial were previously analyzed in Farmanguinhos, Fiocruz, for quality and dosage. The administration of medication was supervised in the residence of patients by a member of the CEPEM staff. Before starting the treatment, blood samples were collected for preparing thick and thin blood films and for parasite DNA extraction. Parasite counts were performed every 24 hours until clearance of parasitemia and then, on days 7, 14, 21, 28, 35, 60 and 90 post treatment.

When possible relapses occurred during the follow up, a new blood sample was collected for parasite DNA extraction allowing comparison of original and relapse parasites. Comparison was performed by PCR amplification of the P5 fraction of the PvMSP-1 gene of both samples using the Single-strand conformation polymorphism (SSCP) technique.

Until now, 66 patients have been submitted to the therapeutic trial and followed for a minimum period of 30 days, while 35 patients concluded the follow up of 90 days. In none of them it was observed signals of chloroquine resistance of the respective *Plasmodium vivax* parasites. Eight possible relapses were observed among patients, from which 7 belonging to schedule B and 1 to schedule A . The mean time of relapses was 65.1 days ± 7. Analysis of MSP-1 alleles of original and corresponding relapse strains of parasites are in course now.

RT16 - MOLECULAR MARKERS FOR DRUG RESISTANCE IN *PLASMODIUM FALCIPARUM* BRAZILIAN ISOLATES

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The prevalence and severity of drug resistant malaria is emerging very rapidly in the Amazon basin of Brazil. In support of clinical trials, we are performing in vitro drug sensitivities, molecular characterization of parasite populations using pfmdr1, cg2 , DHFR and DHPS as drug resistant molecular markers. Sequence analysis of the *pfmdr1* gene revealed Asn, Phe, Cys, Asp and Tyr in the positions 86, 184, 1034, 1042 and 1246 respectively. These point mutations were similar to that previously described in other Brazilian isolates. Southern blot analysis revealed no amplification of the *pfmdr1* gene. These results suggest that three different mechanisms for drug resistance exist for chloroquine, mefloquine and quinine.

A recent publication demonstrates a strong correlation between a specific set of polymorphisms in the cg2 gene of *Plasmodium falciparum* strains from Southeast Asia and Africa, and chloroquine resistance. The data on the only South American strain, 7G8 presented in this paper shows a different set of polymorphism. Preliminary analysis of 18 freshly cultured stables *P. falciparum* isolates from the Brazilian Amazon region showed in vitro resistance to chloroquine and quinine. The cg2 repeats and mutation pattern were similar to the South American strain 7G8. This data could indicate, which corroborates with the *pfmdr1* gene mutation results, that chloroquine resistance in the Brazilian Amazon is a clonal expansion of a single resistant parasite and could point to a different mechanism of resistance in South American strains. In order to complete the analysis, chloroquine sensitive strains from the Brazilian Amazon region were analyzed and cg2 and pfmdr1 gene of the isolates were compared with the resistant parasites.

RT16 - MOLECULAR MECHANISMS OF DRUG RESISTANT MALARIA: NEW TOOLS FOR SURVEILLANCE?

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Plasmodium falciparum resistance to the chloroquine and the antifolate antimalarial drugs is widespread in South America and in Southeast Asia, and is rising in Africa. *In vitro* resistance to the antifolates is determined by mutations in parasite dihydrofolate reductase HFR) and dihydropteroate synthase (DHPS). The role of DHFR and DHPS mutations in therapeutic failure of antifolate antimalarials is less clear. This presentation will summarize data from molecular epidemiological surveys, studies of *in vivo* selection of mutant alleles by drug treatment, and prospective studies of the ability of mutation-specific assays to predict clinical outcomes. The potential use of these assays for surveillance of resistance will be discussed. Preliminary data will also be presented from ongoing studies of associations between in vivo chloroquine resistance and molecular markers for chloroquine resistance, including cg2 and pfmdr1.

RT16 - PURINE TRANSPORT AND SALVAGE IN PROTOZOAN PARASITES

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The purine salvage pathway of protozoan parasites offers a plethora of attractive targets for the therapeutic manipulation of parasitic diseases, as all protozoan parasites are incapable of purine nucleotide biosynthesis again. Thus, protozoan parasites are obligatory scavengers of host purines, and each parasite genus has evolved a unique collection of purine salvage enzymes for purine acquisition. This laboratory has employed an interdisciplinary strategy to dissect the purine salvage pathway of *Leishmania donovani*, the causative agent of visceral leishmaniasis. Amalgamating tools and techniques from molecular biology, biochemistry, genetics, cell biology, immunocytochemistry, structural biology, and computational chemistry, the purine salvage pathway of this parasite has been thoroughly analyzed. Mutational and biochemical studies initially revealed that the key components of the purine salvage pathway of *L. donovani* are the two membrane associated nucleoside transporters, LdNT1 and LdNT2, and

the three intracellular phosphoribosyltransferase enzymes, hypoxanthine-guanine phosphoribosyltransferase (HGPRT), adenine phosphoribosyltransferase (APRT), and xanthine phosphoribosyltransferase (XPRT).

The genes encoding LdNT1, LdNT2, HGPRT, APRT, and XPRT have all been cloned and sequenced, and each has been overexpressed either in L. donovani or in *Escherichia coli* for functional characterization and/or biochemical analysis. In addition, mutants deficient in LdNT1 or LdNT2 transport activity and Ähgprt and Äaprt null mutants have been constructed and characterized. These mutant and knockout strains have provided a further evaluation of purine gene and protein function in intact parasites. Finally, high resolution 3-dimensional structures (<2.0*) of the *Toxoplasma gondii* hypoxanthine-uanine-xanthine phosphoribosyltransferase, *L. donovani* APRT, and *T. gondii* uracil phosphoribosyltransferase in multiple different mechanistic states have also been determined by X-ray crystallography. These structures provide valuable insights into enzyme function and serve as a cornerstone for structure-based drug design and discovery approaches for the treatment and prophylaxis of parasitic diseases.

RT17 – MACROPHAGE AND PROTOZOA INTERACTIONS

RT17 - ISOLATION AND STRUCTURAL ANALYSIS OF THE GPI-ANCHOR FROM *TRYPANOSOMA CRUZI* TRYPOMASTIGOTE MUCINS THAT INDUCES THE SYNTHESIS OF PROINFLAMMATORY CYTOKINES AND NITRIC OXIDE BY MURINE MACROPHAGES

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In recent studies we have shown that mucin-like glycoproteins from *Trypanosoma cruzi* cell-derived trypomastigotes, but not those from epimastigotes or metacyclic-trypomastigotes, were potent inducers of the synthesis of proinflammatory cytokines (IL-12, TNF-a) and nitric oxide (NO) by IFN-g-primed murine macrophages (1,2). Our previous data also indicated that the GPI (glycosylphosphatidylinositol)-anchor alone could be responsible for the macrophage stimulatory activity. Here we report the isolation and partial structural characterisation of the intact GPI-anchor of the mucins purified from epimastigotes and cell-derived trypomastigotes. These and several other purified GPI-containing glycoconjugates were evaluated for their ability to trigger cytokine and NO synthesis by murine macrophages. Briefly, mucins and glycoinositolphospholipids (GIPLs) from trypomastigotes and epimastigotes were purified by sequential organic solvent extraction followed by hydrophobic interaction chromatography (HIC) (3). The intact GPIs were isolated by HIC after exhaustive proteinase K treatment of the native mucins. Purified glyconjugates were assayed for the induction of IL-12, TNF-a and NO by IFN-g-primed peritoneal macrophages from C3H/HeJ (LPS-hyporesponsive) mice, as previoulsy described (1,2). The purified trypomastigote mucin GPI-anchor (tMuc-GPI) showed essentially the same specific activity (relative to *myo*-inositol content) as the intact trypomastigote mucins. tMuc-GPIs induced the synthesis of TNF-a and IL-12 at *myo*-inositol concentrations above 50 pM, and nitric oxide above 5 pM. As expected, the purified epimastigote mucin GPI-anchor (eMuc-GPI) had relatively little or no inducing activity, even at higher concentrations (>10 nM). When analysed by electrospray-mass spectrometry (ES-MS), both purified GPI-anchors showed two ion clusters, corresponding to groups of singly- and, more intense, doubly-charged pseudomolecular ions. In the case of tMuc-GPIs, a complex array of at least 10 doubly-charged ions was observed in the range of m/z 917-1265. In contrast, eMuc-GPIs showed no more than 6 doubly-charged ions in the range of m/z 909-1066. Based on ES-MS and ES-MS/MS (CID) experimental data on the isolated PIs (obtained by nitrous deamination of the GPIs) and also on previous published data (4,5), we could structurally assign all the GPI species observed. In essence, most of tMuc-GPIs are composed of 4-8 hexoses, together with ethanolaminephosphate (EtNP), 2-aminoethylphosphonate (2-AEP) and mainly unsaturated (C18:1 or C18:2) fatty acids in their alkylacyl-phosphatidylinositol (PI) moieties. In contrast, the majority (60%) of the eMuc-GPI species consist of 4-5 hexoses, EtNP, 2-AEP, and fully saturated (C16:0) fatty acids on their alkylacyl-PI moieties. Approximately 40% of the eMuc-GPI species contain an extra 2-AEP residue substituting the EtNP, as previously observed (4). GIPLs purified from both parasite stages had no cytokine/NO inducing activity on macrophages, even at very high *myo*-inositol concentrations (>100 nM). ES-MS and ES-MS/MS analyses showed that ceramide was present as part of the PI moiety in the majority of the trypomastigote GIPLs, akin to most epimastigote GIPL structures reported (6,7,8). Taken together, our results suggest that the potent cytokine/NO inducing activity of trypomastigote mucins can be the result of their unique GPI structural features, *i.e.*, the presence of a higher number of hexoses in the glycan core and an alkylacyl-PI containing mainly unsaturated fatty acids. We are now performing experiments to determine the minimal structural requirement on the trypomastigote mucin GPI-anchor still able to potently stimulate macrophages.

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RT17 - SIGNALING PATHWAYS INVOLVED ON INDUCTION OR REGULATION OF PRO-INFLAMMATORY CYTOKINE SYNTHESIS BY MACROPHAGES EXPOSED TO GPI ANCHORED GLYCOPROTEINS DERIVED FROM *TRYPANOSOMA CRUZI* TRYPOMASTIGOTES

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Induction of IL-12 and TNF-a synthesis by cells from macrophage lineage is a key event on establishing cell mediated immunity (CMI), as well as determining disease outcome in different situations (1). Through the induction of IFN-g synthesis, IL-12 has been shown to be crucial in resistance to multiple intracellular pathogens, including various protozoa (e.g. *Leishmania* sp., *Plasmodium* sp., *Trypanosoma cruzi* and *Toxoplasma gondii*) (2,3,4). However, if uncontrolled the induction of IL-12 by microbial products can lead to a highly toxic immune response, with detrimental effects to the host (2).

In some instances, the IL-12 activity in inducing IFN-g appears to be dependent or potentiated by another monokine, the TNF-a (5). In fact, some studies suggest that the toxic effects of IL-12 are at least in part mediated by TNF-a (2,6). On the other hand, IL-10 has been shown to act as a potent physiologic regulator of IL-12 and harmful immune response elicited by this cytokine (7-9). Thus, defining the signaling pathways involved on induction or regulation of IL-12 synthesis by macrophages, may help defining synthetic products that act either as adjuvants for induction of CMI or inhibitors of ongoing detrimental CMI.

One of the main interest of our research is to identify the biochemical paths involved in the induction or regulation of the pro-inflammatory cytokine synthesis by macrophages exposed to microbial products. As a model we have used IFN-g primed inflammatory macrophages activated with glycosylphosphatidylinositol-anchored mucin-like glycoprotein (GPI-mucin) which is a main membrane component responsible for induction of cytokine synthesis by macrophages exposed to *T. cruzi* trypomastigotes (10-12).

We have initially used key inhibitors for specific signaling pathways and tested their ability to inhibit IL-12 and TNF-a synthesis by macrophages upon stimulation with GPI-mucins (Table). Among the different inhibitors tested, we found that PDTC an inhibitor of induction of NF-kB inhibits both TNF-a and IL-12 synthesis by macrophages(13).

In addition, Cholera Toxin an activator of *adenylate cyclase* was a potent inhibitor of TNF-a and IL-12(p40)

Effects of different inhibitors of protein kinases, adenilate ciclase and NF-KB transcription factor on IL-12 and TNF -a synthesis by macrophages stimulated with protozoan glycolipids

	TNF-a	IL-12(p40)
PDTC (inhibitor of NF-kB)	++	++
Cholera Toxin (activator of adenilate ciclase)	++	++
8-Bromo cAMP (cAMP analogue)	++	++
Dibutyril cAMP (cAMP analogue)	++	++
Pertussis Toxin (inhibitor of adenilate ciclase - Gi)	—	—
SQ-22536 (inhibitor of adenilate ciclase)	—	—
HA (miosyn kinase, PKA)	—	—
SB203580 (inhibitor of p38 MAP-K)	++	+
PD098059 (inhibitor MAPKAP-K1)	—	—
Rapamycin (inhibitor of PKC and HSP-70)	++	—
Ro-31-8220 (inhibitor of PKC and HSP-70)	++	—
H7 (inhibitor myosin kinase, PKC and PKG)	++	—
U-73122 (inhibitor of PKC and hidrolisis of PI - IP3)	++	—
Wortmannin (inhibitor of PI 3-kinase)	++	—
Tyrphostin A126 (inhibitor ofTyrosine kinase)	++	—
Genistein (inhibitor ofTyrosine kinase)	++	—

synthesis. In order to confirm that the inhibitory effect of Cholera Toxin was due to the generation of cAMP we used two cAMP analogues which mimic the action of cAMP *in vivo.* Either 8-bromo cAMP or dibutyryl cAMP were shown to inhibit both TNF-a and IL-12(p40) synthesis by macrophages stimulated with GPI-mucins. So was prostaglandins of the E series (PGE), a physiological stimulator of cAMP generation. Since high intracellular levels of cAMP have been shown to augment IL-10 expression in T lymphocytes and macrophages (14,15), we decided to investigate a possible role for IL-10 on the cAMP modulatory effect on IL-12(p40) and TNF-a synthesis. Our data also show a gradual increase of IL-10 synthesis by macrophages stimulated with microbial products cultured in the presence of increasing concentrations of either Cholera Toxin, 8 bromo cAMP or dibutyryl cAMP. Finally, in order to test the importance of endogenous IL-10, we used macrophages from IL-10 knockout mice. Interestingly, we found that cAMP inhibited microbial stimuli-induced IL-12(p40) but not TNF-a synthesis in macrophages lacking a functional IL-10 gene.

Among different inhibitors for protein kinases we found that SB203580, a pyridinil-imidazole compound and specific inhibitor for p38 MAP kinase (16,17), is also potent inhibitor of TNF-a and in a less extend of IL-12(p40) synthesis by inflammatory macrophages exposed to either GPI-anchored mucin like glycoproteins (tGPI-mucins) or purified GPI (tGPI) anchors isolated from *T. cruzi* trypomastigotes. In contrast, PD098059 and inhibitor of MAPKAP-K1 (MAPK-1 pathway) (18) had no inhibitory effect on IL-12(p40) or TNF-a synthesis by macrophages expose to different microbial stimuli. We also measured the activity of downstream targets of p38 MAP kinase and MAPKAP-1 before and after macrophage stimulation with either tGPI-mucins or tGPI. Our results show that tGPI activates both MAPKAP-K1 and MAPKAP-K2. However only the MAPKAP-k2 activity was inhibited by the compound SB203580. The peak of MAPKAP-K2 activity induced by tGPI was at 15 min post macrophage simulation. The ability of IFN-g and TNF-a to potentiate or induce p38 MAP kinase activation was also evaluated. IFN-g but not TNF-a was shown to significantly augment the activity of MAPKAP-K2 induced by either tGPI or LPS. We also studied the ability of different compounds (i.e. cAMP analogues, dexametazone and IL-10) known to inhibit the synthesis of IL-12(p40) and TNF-a, in their ability to inhibit MAPKAP-K2 activation induced by tGPI or LPS in the presence or absence of IFN-g. Our results show that cAMP analogues and IL-10 had no modulatory effect on MAPKAP-K2 activity. In contrast, dexametazone had a partial but consistent inhibitory effect on MAPKAP-K2 activity elicited by the different microbial stimuli. These studies indicate the involvement of p38 MAP kinase pathway on the induction of TNF-a and IL-12 synthesis by macrophages exposed to GPI anchors derived from *T. cruzi* trypomastigotes.

In addition to SB203580, various other inhibitors of protein kinase C (19,20) and tyrosine kinase (21) blocked the TNF-a but not IL-12(p40) synthesis by macrophages stimulated with the protozoan glycolipid plus IFN-g. Among these inhibitors we can mention that Rapamycin and Ro-31-8220 known as PKC inhibitors, which block the p70 S6 kinase that is responsible for phosphorylation of HSP 70, another downstream product from one of the MAP kinase pathways (22). In addition, the H7 was shown to inhibit the TNF-a synthesis in the similar doses required to inhibit PKC (20). Among the drugs which were found to inhibit TNF-a we also found U-73122 (block PLC and hydrolysis of PI - IP3) Wortmannin (block PhosphatidylinositoI 3-kinase) (PI 3 kinase)and Tyrphostin A126 as well as Genistein (inhibitors of Protein tyrosine kinase). The identification of compounds that can be used in vivo, and that inhibit induction and detrimental effects of TNF-a but not IL-12 maybe of interest. The understanding of the mechanisms controlling of macrophage stimulation by microbial products may suggest novel ways of controlling parasite-induced immunopathology.

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RT17 - LYMPHOCYTE APOPTOSIS: A DOUBLE-EDGED SWORD IN HOST CELL-MEDIATED IMMUNITY AGAINST *TRYPANOSOMA CRUZI*

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The role of lymphocyte apoptosis in the control of *Trypanosoma cruzi* replication in macrophages was investigated in a culture system. Infected macrophage monolayers controled parasite replication after addition of rIFN-γ or supernatants from activated CD4+ T cells. Surprisingly, parasite replication was greatly exacerbated when anti-TCR activated CD4+ T cells from infected mice were cultured with the monolayers. Blocking activation with anti-LFA-1 also reduced parasite replication. Exacerbated parasite growth required cell contact between T cells and macrophages. Growing parasites were infective trypomastigotes by morphological criteria.

Activation-induced cell death (AICD) is a prominent response of these CD4+ T cells following antigen receptor (TCR) stimulation (1). The involvement of AICD in exacerbated parasite replication was demonstrated by the following criteria: (i) a pro-apoptotic T-cell stimulus (TCR) exacerbated parasite growth, while a non-apoptotic stimulus (Ly-6) had no effect; (ii) mimicking AICD by Ly-6 T-cell activation coupled to anti-Fas treatment, exacerbated parasite replication; (iii) blocking T-cell AICD with anti-FasL mAb also reduced parasite replication (2); (iv) activated CD4+ T cells from FasL-deficient *gld* mice failed to exacerbate parasite growth in wild-type macrophages. Neither anti-Fas or anti-FasL had any effect on infected macrophages alone. Anti-Fas treatment markedly reduced (by 70%) activation-induced IFN-γ secretion by CD4+ T cells from infected mice, in spite of killing only 25-30% of the cells (2). These results demonstrate that AICD is responsible for the observed exacerbation in parasite growth following T-cell activation. Presumably, one effect of AICD is a marked reduction in the amount of IFN-γ available for macrophages.

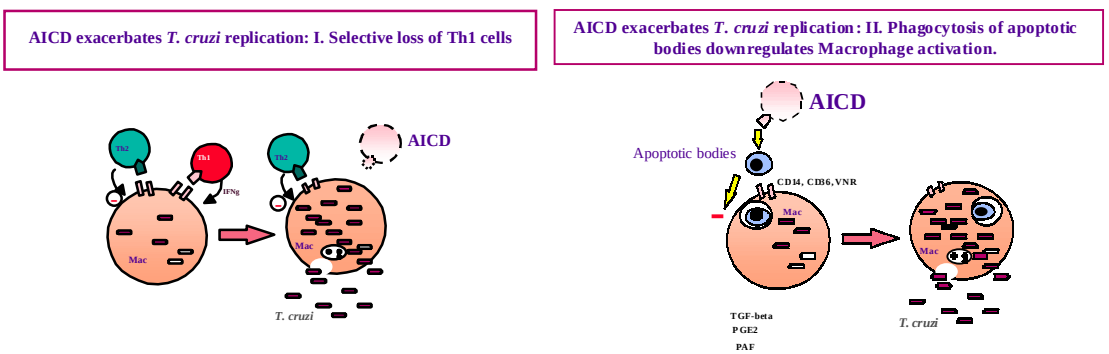

Fig.1. Lymphocyte apoptosis exacerbates *T. cruzi* growth: 2 models.

However, the need for physical contact between T-cells and macrophages in this system (2), still requires explanation. One possibility is that Th2-type T cells selectively survive AICD and physically interact with macrophages to deactivate them (Fig.1-left). This possibility is under investigation. The second and non-exclusive possibility, is that phagocytosis of apoptotic T cells directly deactivates macrophages (Fig.1-right). Recently, we have obtained evidence for this latter concept. Phagocytosis of apoptotic, but not necrotic T cells, increased trypomastigote replication in macrophages, although not to the same extent as "live scene" AICD. Moreover, *in vivo* injection of apoptotic T cells exacerbates parasitemia in infected animals. Apoptosis has a clear host-protective, general anti-inflammatory effect. However, our results indicate that several distinct anti-inflammatory effects of lymphocyte apoptosis also help parasite persistence in *Trypanosoma cruzi* infection.

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RT17 - *IN VITRO* MODULATION OF HUMAN MACROPHAGE LEISHMANICIDAL CAPACITY

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At present, the outcome of an infection with *Leishmania* is considered to depend on the preferential action of Th1 (IFN-g, TNF, IL-12) or Th2 cytokines (IL-4, IL-10, TGF-b), resulting in protection or disease progression, respectively (1-2). By virtue of its capacity to induce leishmanicidal activity in macrophages, IFN-g has been extensively studied as the protagonist among Th1 cytokines. In contrast, few data are available on type I IFN (IFN-a/b), but a protective role, correlated to NO synthase induction, has been demonstrated in murine leishmaniasis (3-4). In human cells, however, IFN-b has been shown to block IFN-g induction of MHC class II (5-7) and FcgRI (8). Therefore, we examined the possible effect of IFN-b in *Leishmania*-infected human macrophages, derived from peripheral blood of healthy donors.

We were able to show that treatment of *L. amazonensis* and *L. braziliensis*-infected macrophages with IFN-b strongly increased cellular parasite load in a time- and dose-dependent manner. When both IFNs were added simultaneously, IFN-b was able to completely antagonize the protective effect of IFN-g. Due to this negative role in *Leishmania*-infected human macrophages, we believe IFN-b might be considered as a potential Th2 type cytokine, in contrast to murine IFN-a/b, indicating a strong difference between species, as has been observed for NO production as well.

In spite of the large amount of literature dedicated to the importance of zinc to the immune response *in vitro* and *in vivo* in numerous infectious and non-infectious diseases, no data exist on the possible role of zinc (Zn^{2+}) in leishmaniasis. Nevertheless, zinc has been shown to induce Th1 cytokines, such as IFN-g and TNF-a. Accordingly, we investigated the possible effect of zinc supplementation *in vitro* on the immune capacity of human macrophages infected with different *Leishmania* species. Our results show that addition of zinc (as $ZnCl_2$) to macrophages infected with *L. amazonensis*, *L. braziliensis* or *L. major* dramatically decreased intracellular parasite load in a time-dependent manner. Reduction in parasite load was maximal at 10 mM of Zn^{2+} and decreased at higher concentrations. This optimal concentration corresponds to normal serum zinc levels, pointing at a possible negative role for zinc deficiency in leishmaniasis, as suggested recently in cutaneous leishmaniasis patients in Turkey (9). The molecular mechanism through which zinc reduces intracellular parasite burden is currently under investigation, but its interaction with the cytokine network is strongly suspected.

In line with our previous data, these results demonstrate that IFN-b can be considered as a specific antagonist and zinc as an adjuvant of IFN-g action, even in a more complex biological function, namely the clearance of intracellular *Leishmania* parasites from infected human macrophages. Our actual challenge is to explore the possible physiological relevance of these findings at the *in vivo* level.

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RT18 – VECTORS' BIOLOGY

RT18 – CURRENT TRANSMISSION OF CHAGAS DISEASE IN THE STATE OF CEARÁ, BRAZIL

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After the success of the *Triatoma infestans* control, the states of the Northeast contribute now with 65% of the current transmission of the human disease in Brazil, being the *Triatoma brasiliensis* and the *Triatoma pseudomaculata* the species more frequently captured by the Chagas Disease Control Program. Original of the caatinga, *T. brasiliensis*

occupies different silvatic habitats, especially associated to rodents and stones. In spite of the domestic population would be eliminated through methodology similar used against *T.infestans*, the houses are recolonized for the triatomines coming from the peridomestic or wild populations. *T. pseudomaculata* is captured under peels of dry trees. Predominantly peridomiciliar, in some areas substituted *T. brasiliensis* in treated areas with insecticides.

In this work we correlated the results of a serological inquiry accomplished in the municipality of Independência (5:23'S and 40:18'W), State of Ceará, with the food sources, identified by the precipitine reaction. In the period from Jan/96 to Aug/97 the domicilary units infested was 27.4%, being 10.8% indoors. The serological inquiry included 2450 people. The blood was collected in filter paper, and processed by the imunofluorescence and hemaglutination. The rates of global prevalence and for age group were:

Total	0-10 years	11-15 years	16-20 years	21-30 years	31-40 years	41-50 years	>50 years
5.7%	0.8%	2.3%	4.9%	5.6%	7.0%	10.6%	12.6%

The 0-10 year-old age group included 657 children, having been five positive. In the intradomiciles 50% of the blood ingested samples by *T. brasiliensis* revealed to be of human origin, being especially birds, rodents and dogs important in the peridomicile. Rare *T. pseudomaculata* were captured inside of the houses, and in the peridomicile this species presented great association with birds and rodents. These data demonstrate the persistence of human transmission of Chagas disease in the Northeast, in spite *T. infestans* is in roads of being eradicated of Brazil

RT18 - APPLICATION OF TRIATOMINE SALIVA AS A POPULATIONAL MARKER

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The genus *Rhodnius* currently includes 13 species widely distributed in South and Central America and recognized based on morphological features. Distinction between *Rhodnius* species is of considerable importance because of their different epidemiological significance as vectors of *Trypanosoma cruzi* (WHO 1991). *R. prolixus* is widely distributed in Venezuela, Colombia, and parts of Central America, where it is an important domestic vector of *T. cruzi*, causative agent of Chagas disease (Schofield & Dujardin 1997). It is morphologically similar to *R. robustus* which frequently colonizes palm tree crowns in the same regions, as well as in parts of Ecuador, northern Peru and Brazil (Lent & Wygodzinsky 1979). Both species are also similar to *R. neglectus* from central Brazil and *R. nasutus* from northeastern Brazil, so that the four species are often described as the '*prolixus* group' (Barrett 1988).

We compared salivary heme proteins profiles of eight *Rhodnius* species: *R. prolixus* (Honduras, Venezuela and Brazil population), *R. robustus*, *R. neglectus*, *R. nasutus*, *R. ecuadoriensis*, *R. pallescens*, *R. pictipes* and *R. domesticus*. Salivary glands from each insect were dissected out, washed in 0.9% NaCl and transferred to 5 ml of running buffer on a microscope slide in a humid chamber where they were maintained until all insects had been prepared. The prepared glands from each insect were then disrupted with dissection needles, and the salivary contents applied to a starch gel electrophoresis plate. Electrophoresis was carried out at 300V for 150 min. The running buffer was 0.15M glycine/NaOH, pH 9.5; the gel buffer was the same but diluted 1:10. After electrophoresis, the gels were immersed for 10 min in a staining solution of 0.3 mg/ml tetramethylbenzidine in a 1:1:1 mixture of ethanol, acetic acid and water. They were then transferred to 2% hydrogen peroxide until the bands began to develop.

It was possible to distinguish all species studied with this technique. This approach also distinguished *R. prolixus* from *R. robustus* and *R. neglectus* from *R. nasutus*, species with extreme phenotypical similarity. We also compared heme proteins profiles of *R. prolixus* from three different laboratory colonies constructing a phenogram (UPGMA) based on shared bands using the Dice similarity coefficient (Dice 1945). The Honduran and Venezuelan populations could not be distinguished from each other, but the Brazilian population was well separated from the others. The high similarity between Honduran and Venezuelan specimens lends support to current theories that the Central American populations of *R. prolixus* may have a Venezuelan origin.

Panstrongylus megistus is an important species in the epidemiology of Chagas' disease in Brazil because of its high susceptibility to infection by *T. cruzi*, wide geographical distribution and adaptability to a range of habitats and hosts, which allows it to colonize both domestic and peridomestic ecotopes. Nevertheless, populations of this species from different regions show behavioural variation in their capacities to colonize the domestic environment. In northeastern Brazil *P. megistus* generally occurs in man-made habitats such as those encountered in the Recôncavo Baiano region, where repeated attempts to collect this species in sylvatic habitats have been fruitless (Forattini et al. 1977).

Triatomines were collected from the following localities: Campo Formoso, Bahia (BA); Belo Horizonte, Minas Gerais (MG) and Florianópolis, Santa Catarina (SC). Laboratory colonies were established from wild-caught specimens and the F1 (BA and MG) or F3 (SC) generations of these colonies used in analyses. Saliva was obtained by electric stimulation of adult insects. Electrophoresis (SDS-PAGE) of triatomine saliva was carried out on a 12.5% polyacrylamide slab gel and the protein bands were stained by Coomassie blue (G-250). Approximately 5 mg of protein were loaded in each lane. Estimates of the relative amount of protein in the bands of each electrophoretic profile were made by densitometry using the Is-1000 Digital Imaging System (Alpha Innotech Corporation, San Leandro, CA).

The electrophoretic analysis of saliva of individuals of *P. megistus* showed a complex protein composition with molecular weight ranging from 9 to 87 kDa. The most characteristic protein band profile was observed in the region between 20 to 24 kDa. This region contain 53.8 ± 10.1% (n = 60) of the total applied protein distributed in 2 to 4 bands as determined by densitometry. Remarkable differences were seen between the band profiles of the SC and BA populations. All SC individuals showed one 20 kDa and another 23 kDa protein band, while BA individuals did not show the 23 kDa protein band and only two of them (10% of BA analyzed individuals) showed the 20 kDa band. Seventeen of the MG individuals (85%) showed a band of 23 kDa and 14 (70%) one of 20 kDa. The phenogram (UPGMA) constructed on the basis of bands that presented ^{3} 5% of the total applied showed the existence of two main groups, the SC population being completely separated from that of BA. The MG individuals could be distributed between the two groups, with 14 (70%) in the SC group and 6 (30%) in the BA one. The number of bands analyzed varied from 4 to 9 per insect. With respect to this parameter, the BA individuals with 5.0 ± 0.9 bands were different from those of SC and MG with 6.6 ± 1.1 and 6.1 ± 1.1, respectively ($p<0.01$). There was no significant difference in band number between the MG and SC populations ($p = 0.12$).

The saliva of hematophagous insects possesses pharmacologically active substances that assist in blood feeding (Ribeiro 1987) and also modulate feeding success through immunogenic reactions (Lehane 1991). If variation in the triatomine saliva (protein composition and/or heme protein profiles) as demonstrated here is a consequence of selection favouring a better adaptation to local hosts, it may change faster than neutral characters, as shown by techniques as isoenzymes and morphometrical features. Therefore saliva variation may represent a good genetic marker for vector population studies.

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RT18 - CONTROL OF TRIATOMINAE IN LATIN AMERICA

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Accumulated experience throughout Latin America – especially in the Southern Cone countries and in parts of Central America and the Andean Pact – clearly demonstrates the feasibility of halting Chagas disease transmission through elimination of domestic populations of Triatominae (Schofield & Dias 1998) supported by measures to reduce the risk of transfusional transmission in bloodbanks (Dias & Schofield 1998). Uruguay has already been certified free of transmission, and it is expected that other countries of the Southern Cone will follow this success during the next few years.

The experience shows that large-scale spraying of houses in infested localities with residual formulations of synthetic pyrethroids can be highly effective in eliminating domestic populations of Triatominae – mainly *Triatoma infestans* in the Southern Cone, and *Rhodnius prolixus* in parts of Central America and the Andean Pact. From this success however, we face the problem of reinfestation of treated localities. Where this is due to the original species, genetic and phenetic markers have been developed to distinguish between true reinfestations, due to bugs immigrating from untreated houses, and apparent reinfestations due to bugs surviving the initial treatment (Dujardin et al. 1996 1997a, b), which provide a useful guide for operational decisions. But in the case of reinfestation by secondary species the problem is more complex, both in operational terms and also in terms of applicable techniques of entomological surveillance and control. Consider for example, the finding of bugs in a peridomestic habitat. Should this be considered of equal importance to a domestic infestation, requiring immediate retreatment? In the case of *T.*

infestans this would seem an appropriate decision, but perhaps not in the case of other species such as *T. sordida* which frequently colonises chicken houses and only rarely seems to invade human dwellings (cf. Diotaiuti et al. 1995). In Central America and the Andean Pact, this problem is particularly acute for the control of *T. dimidiata*, which has extensive silvatic, peridomestic and domestic populations. We face the question of deciding when and how to respond to peridomestic infestations, and also of which techniques to apply. In peridomestic habitats, available techniques of residual spraying are of reduced effectiveness, and newer techniques – such as that of 'xenointoxication' using pour-on formulations – are yet to be field tested.

We also face questions related to land-use changes throughout Latin America. The Triatominae have been poetically described as "a parade of species queueing up to colonise rural dwellings" and it seems that ecological changes frequently lead to important adaptations of otherwise silvatic Triatominae previously considered of little epidemiological relevance. Recent literature shows a growing number of reports of little-known silvatic species adapting to peridomestic and domestic situations – including *T. rubrovaria* in Uruguay and Rio Grande do Sul, *T. costalimai* in Goiás, *Panstrongylus geniculatus* in Pará and southern Venezuela, *P. rufotuberculatus* and *Eratyrus mucronatus* in Bolivia, and various species of *Rhodnius* in Amazonia (Valente & Valente 1993, Coura et al. 1994, Noireau et al. 1994, 1995, Aguilar & Yépez 1996, Dujardin et al. 1998). As the transition procedes we may eliminate the domestic populations, but we should also develop ways to monitor and inhibit the domestic adaptations – which is difficult since we do not yet understand the adaptive process. In general terms we can imagine that, for example, deforestation may provoke host mortality leading to reduced nutritional status of silvatic bugs, which may then disperse in search of new feeding sites. But what is the underlying adaptive mechanism, and what genetic changes are involved? Most importantly, what are the best indicators that can be used in entomological surveillance, and at what point could we consider specific control interventions?

Some researchers have commented – perhaps not always in jest – that the current Chagas control initiatives may leave them short of research material. This is far from reality. The control initiatives show the value of research, giving a clear justification for what has already been achieved. But they also offer important opportunities for new research, especially to understand little-known species of Triatominae whose potential epidemiological significance may still be underestimated. It may be that research laboratories in the future will give less emphasis to the well-studied *T. infestans* and *R. prolixus*, concentrating on more difficult species and applying a wide range of modern techniques to understand their adaptive processes, leading, eventually, to the operational tools required by the changing nature of national surveillance and control services.

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RT18 - PHYLOGENY OF TRIATOMINAE. THE *TRIATOMA INFESTANS* COMPLEX

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Hemiptera seem to derivate from Polyneoptera, with fossils found since the early Permian but probably with ancestors existing during the Devonian. Apparently, common ancestors of Homoptera and Heteroptera were sap-

suckers, feeding behavior maintained by the first of these suborders. Modifications of the buccal structure were necessary for adaptations to the predatory condition first, and to hematophagism later. Predators needed to modify stylets to perforate some tissues. Hematophagous Reduviidae needed new modifications of the stylets for other kind of tissues, to lose the peritrophic membrane developing a microvillar membrane for the efficient absorption of solutes (Terra 1988). Furthermore, they need to find the feeding source, both the host and the fluid (blood), with sensilla located in different areas of their anatomy, specially on antennae and rostrum (Catala 1998). Modifications in the saliva were necessary for hematophagism, too. Predator have a proteolytic saliva while in hematophagous saliva does not have a digestive function and biting should be as painless as needed to complete the blood intake. Other aspects of hematofagism are the fast compensation of the great amount of ingested blood, through an inmediate excretion of water and salts, and the hemolysis of the erythrocytes through a hemolytic factor (Azambuja et al. 1983).

Lent and Wygodzinsky (1979) think that the origin of Triatominae is monophyletic, based in several apomorphic traits commonly found in most of the species. However, they recognized that the external genitalia are of plesiomorphic structure in the less specialized forms (Triatomini), a fact not useful for the analysis of relationships. For comparisons of male genitalia between genera, see Jurberg et al. (1998). Lent and Wygodzinsky (1979) pointed out the importance of the third rostral segment taking the upward position when feeding and consider that the possible "sister group"could be Physoderinae, according to information published by Carcavallo and Tonn (1976). The origin of Triatominae has more possibilities to be polymorphic taking into account the combination of plesio and apomorphic factors separating the five tribes and seventeen genera, and the geographical distribution, typical for some groups. *Lynchcosteus* Distant, 1904 is found only in India; *Triatoma rubrofasciata* De Geer and some related species are found in other regions out of the Americas but most of the species of this genus are American. But *Triatoma* Laporte, 1832 has great differences according to some areas, with especific complexes: *T. phyllosoma* complex (5 known species) and *T. protracta* complex (3 species and probably several subspecies) are typycal of North America, *T. flavida* complex (3 species) are characteristic of the Caribbean (Cuba and Jamaica), *T. infestans* complex, *T. breyeri* complex and *T. sordida* complex are prevalent in South America. Genus *Dipetalogaster* Usinger, 1939 is found only in Lower California, *Bolbodera* Valdés, 1910 is typical of Cuba, *Paratriatoma* Barber, 1938 is from North America and the two species of Parabelminus Lent, 1943 are restricted to the Atlantic Tropical Forest (Mata Atlántica) between Bahia and Rio de Janeiro. *Eratyrus* Stal, 1859 has a large distribution between Mexico and Bolivia. However, *E. cuspidatus* is found West to the Andes and *E. mucronatus* only on the East of that orographic chain. This could be explain with a speciation process after the orogenic movements at the end of the Tertiary or begining of the Quaternary; furthermore, the existence of *E. cuspidatus* in Central America and Mexico should occur after the emergence of the Central American isthmus that started on the Quaternary.

There are some stenophagous species with almost obliged relationships with some host as feeding sources and this could be an ancient behavior. *Psammolestes* (three species) is related with birds Dendrocolaptidae; *Cavernicola pilosa* feeds on bats; *Microtriatoma trinidadensis* is related with biocoenosis of epiphyte Bromeliaceae; *Panstrongylus geniculatus* follows the geographical distribution of armadillos of the family Dasypodidae, from Mexico to Argentina; *T. protracta* has a strong relationship with rodents of genus *Neotoma*.

The polyphyletic origen permits the explanation of the morphologic differences and the similarity with insects belonging to other subfamilies. The tribe Alberproseniini, with reduced anteocular region, very large eyes and flattened body resembles certain Cetherinas, as *Homalocoris varius* Perty. *Psammolestes* has several characters of the second South American genus of Physoderinae, *Harpinoderes* Martínez & Carcavallo, 1988. Some species of the tribe Bolboderini are so similar to Reduviinae, i.e. *Aradomorpha championi* Lent & Wygodzinsky, 1944, that, as summarizes Schofield (1995): *"This species of Reduviinae is so similar to Triatominae that it was at one time erroneously classified as a new species of Microtriatoma (Sherlock & Guitton 1982)"*.

Taking *T. infestans* complex as an example, it is generally agreed that the specific origin could be in Bolivia, somewhere near Cochabamba (Usinger et al. 1966). However, this concept cannot explain the long distances separating the others species of the complex. One hypothesis that could fill all the gaps is that one of the species or an ancestor dispersed from the Subtropical Dry Forest, Chaco (SW Paraguay, SE Bolivia, N Argentina). From there, one line went to the North and Northwest, with feeding association to Caviidae, and through the domestication of these rodents by indians, the insect was introduced to the human habitats long ago and adapted easily to the optimal environmental conditions; this is *T. infestans s. str.*, that later was dispersed by several countries by passive way, with human migrations. It is possible that some populations of this line could remain under wild conditions, following a different speciation in the same geographical area but in other habitats (Noireau, pers. commun.). Other line, ornithophylous, went to the south, separating later according with feeding preference: Psitacidae or exceptionally Dendrocolaptidae, *T. delpontei;* other birds but never Psitacidae, *T. platensis.* Other line went to the South East, crossing the Parana and the Uruguay rivers and later following to the North, and selecting places with stones, with feeding sources consisting in reptiles and rodents in the rupestrial environment, *T. rubrovaria.* The last, went to the Est, entering in the subtropical forest with high humidity and living in hollow trees and under bark, taking advantage of its black color to hide and survive, *T. melanosoma*, sometimes found on the surroundings of human dwellings.

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RT19 - PROTOZOA CELL BIOLOGY

RT19 - STRUCTURE AND FUNCTION OF GLYCOSOMAL MEMBRANE PROTEINS OF *TRYPANOSOMA BRUCEI*

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Kinetoplastid protozoa confine large parts of glycolysis within glycosomes, which are microbodies related to peroxisomes. We cloned the gene encoding the second most abundant integral membrane protein of *Trypanosoma brucei* glycosomes. The 24kDa protein is very basic and hydrophobic with two predicted transmembrane domains. It is targeted to peroxisomes when expressed in mammalian cells and yeast. The protein is a functional homologue of Pex11p from Saccharomyces cerevisiae: pex11• mutants, which are defective in peroxisome proliferation, can be complemented by the trypanosome gene. Sequence conservation is significant in the N-terminal and C-terminal domains of all putative Pex11p homologues known, from trypanosomes, yeasts and mammals. Several lines of evidence indicate that these domains are oriented towards the cytosol. TbPex11p can form homodimers, like its yeast counterpart. The TbPEX11 gene is essential in trypanosomes. Inducible overexpression of the protein in *T. brucei* bloodstream forms causes growth arrest, the globular glycosomes being transformed to clusters of long tubules filling significant proportions of the cytoplasm. Reduced expression results in trypanosomes with fewer, but larger, organelles.

The most abundant glycosomal membrane protein has a molecular weight of 26kd and is also basic, hydrophobic, and able to form dimers. The function of this protein is now being investigated.

RT19 - KINETOPLAST DNA STRUCTURE AND REPLICATION IN *TRYPANOSOMA CRUZI*

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Kinetoplastid parasites replicate their mitochondrial DNA (kDNA) by one of two very different mechanisms, exemplified by those of *C. fasciculata* and *Trypanosoma brucei.* In *C. fasciculata*, newly synthesized minicircles are distributed uniformly around the network periphery during the replication process, whereas in *T. brucei*, they are found only in antipodal positions at the network edge. We have recently developed a rapid, accurate and vivid technique for determining the mode and stage of replication of every network within large populations of isolated kDNA from any parasite. The method involves selective incorporation of a fluorescent nucleotide into the gaps specific to newly replicated minicircles. The pattern and distribution of fluorescence obtained reflects the distribution of newly replicated minicircles within the network and is diagnostic of the underlying replicative mechanism. Using this method, we have shown that *T. cruzi* epimastigotes replicate their kDNA by a mechanism similar to that of *C. fasciculata.* Further, we found that the structure of networks in different stages of replication to be very similar in epimastigotes and amastigotes. Unexpectedly, the structure of networks in trypomastigotes is very different. Using the fluorescein incorporation technique, all trypomastigote networks incorporate label only very weakly, but uniformly across the entire network surface area. This indicates that minicircles in trypomastigote networks are almost all covalently closed, and that the networks are all in a non-replicative state. EM analysis also shows that trypomastigote networks are much smaller in surface area than those of amastigotes and epimastigotes, although in thin section EM trypomastigote kDNA appears larger due to a different cellular organization. In addition, EM analysis shows that the minicircle density within trypomastigote networks is very much higher, indicating a distinct network topology. Studies are in progress to determine whether the changes observed in trypomastigote networks are due to changes in network topology, minicircle copy number, or both.

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RT19 - THE PROCESS OF PRIMITIVE MITOSIS IN *TRITRICHOMONAS FOETUS*

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The process of mitosis has undergone evolution. This change most probably was accompanied by an increase in genome complexity and the compartmentalization event (Pickett-Heaps 1974, Cavalier-Smith 1991). Contrary to what happens in higher eukaryotes, where the nuclear breakdown occurs in the beginning of cell division, in the closed type of mitosis the nuclear envelope is maintained totally integral. The nuclear division may be "closed" or "semi-opened" or "opened" depending in the degree of nuclear envelope persistency (Heath 1980). The closed type of mitosis with extranuclear spindle is characteristic of dinoflagelates (Perret et al. 1993) and trichomonads (Brugerolle 1975). In this system, the nuclear envelope stands in between the extranuclear spindle and the chromossomes. How then microtubules segregate chromossomes? Several authors sugest kinetochores anchored on the nuclear envelope do this link (Kubai 1975, Heath 1980, Cavalier-Smith 1993). In an attempt to explore the primitive closed mitosis process we used as cell model *Tritrichomonas foetus.* Our results are in disagreement with these previous reports in relation to the kinetochore structure/function and also in the description of the behavior of the cytoskeleton during morphogenesis (Juliano et al. 1986, Viscogliosi & Brugerolle 1994, Delgado-Viscogliosi et al. 1996). Molecular phylogeny indicates that this organism is among the most early diverging eukaryotes to be studied to date (Cavalier-Smith & Chao 1996). This protist is a parasite from the urogenital tract of cattle. It belongs to the Parabasalia group, which harbors amitochondrial protists most commonly found as parasites or symbionts of wood digesting insects or in the intestine or urogenital tract of a wide variety of vertebrates and invertebrates. *T. foetus* cell division serves as an interesting model to study the mitotic evolutionary path, since it presents a membrane-dependent genomic segregation mode as prokaryotes and also a microtubular spindle apparatus as all eukaryotes.

The closed mitosis presents three sets of microtubules: (1) pole-to-nucleus, which are attached to the nuclear envelope, (2) pole-to-pole microtubules, which forms cytoplasmic channels across the nuclear compartment, (3) pole-to-cytosol. Other dominant features include: the presence of a spindle pole body or a microtubule organizing center to nucleate the spindle and a plane of cytokinesis situated perpendicular to a symmetric, bipolar mitotic apparatus (Kubai 1975). Other variations could include: sophistications of a calcium controlling system, different extents of efficiency and energy consumption. The differences in this mode of mitosis are related to the number of channels throughout the nucleus, dinoflagelates always have more than one and trichomonads only one central channel. Trichomonads skeletal elements are didvided in three main systems. The mastigont system includes the flagella together with their basal bodies and connected rootlet filaments. The costa structure which anchors the recurrent flagellum outstands as the main rootlet filament together with the Parabasal filament (from where the Phylum derives its name) which in turn, supports the Golgi complex. Second, the pelta-axostyle complex is composed of a rigid sheath of parallel arranged microtubules which runs down the axis of the cell body. The third system may be represented by the spindle apparatus which appears only during cell division.

Although the cell cycle in protists is poorly studied, there are evidence that nuclear and cellular divisions differ from typical eukaryotic cells in many aspects (Naysmith 1995). In an attempt to explore the primitive cell division of *T. foetus* we used several approaches. The first step was to obtain a high index of mitotic cells. Therefore, we established a synchronization procedure using hydroxyurea, a drug that is known to block DNA synthesis, arresting vertebrate cells on the G1-S phase boundary (Yarbro 1992). A mid-log culture of *T. foetus* was incubated with Hydroxyurea for 15 hr at a 4mM concentration. Fresh medium, free of HU, was then supplied to the cells. As a result, after 4 to 6 hr of drug removal about half of the population of cells are seen under mitosis. At this time interval cells were collected and prepared to be analyzed by several techniques. Living synchronized cells were followed using video enhanced interference contrast microscopy. Scanning electron microscopy, electron micrscopy serial thin section, 3D computer based reconstruction, Panotico staining and confocal laser microscopy of imunofluorescence with anti-tubulin antibody or of DNA fluorescent stain were used to characterize cell morphogenesis. In addition we also produced a hypothetical animation of the whole morphogenesis using a representation of major skeletal structures and of the nucleus to picture their overall interaction.

Our analyses, based on these combined data, revealed six main morphologies of *T. foetus* along the cell cycle. Interphase cells present the typical tear drop shape with three anterior flagella and one recurrent that runs toward the posterior tip of the cell body, one axostyle, one costa, and one nucleus are found. Still in interphase, a pre-mitosis morphology is characterized by the presence of duplicated skeletal structures including all set of flagella. During mitosis, as the microtubule organizing centers (situated beneath the anterior flagella's basal bodies) starts to nucleate the spindle they separate the doubled skeletal elements apart conferring to the cell continuous shape changes. We characterized morphogenesis in four phases. In addition we correlate the importance of a bilateral and mirror symmetry of the mitotic apparatus with the events of genomic partition and daughter cell disjunction. In principle, we demonstrate that the nuclear partition is in addition to the spindle action, performed with the direct mechanical aid of the axostyle, a stable microtubular structure of this cell's skeletal system. On the other hand we also describe the importance of the mastigont system.

Analyses of the ultraestructure of the dividing cell rendered information about the details of membrane-micro-

tubule interaction and revealed a complex transformation of the intact nuclear envelope. We observed finger-like expansions on the pole regions of the nuclear envelope and the formation of "bubbles" in between its inner and outer membranes. Microtubules in bundlles were observed anchoring onto the nucleus or passing through its interior by a cytoplasmic channel. Concerning organelle redistribution, we could verify the presence of an integral Golgi apparatus on each mitotic cell pole and of the nucleolus, contrary to what happens in higher eukaryotes were they do fragment. The endoplasmic reticulum was observed surrounding the mitotic apparatus, a common feature on other reports of unorthodox types of mitosis that is associated with a calcium controlling function (Wolf 1995).

In the present study we have shown that *T. foetus* mitosis is held not only by the spindle microtubules as in higher eukaryotes but also with the aid of the axostyle and of the flagellar propulsion. Further, we demonstrated alterations of the nuclear envelope which might contribute to realize a still obscure mode of membrane-based chromossome segregation pattern. We propose with this study a better understanding on the evolution of mitosis and we put forward new hypothesis on the segregation mechanism.

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RT19 - PYROPHOSPHATE IS THE MOST ABUNDANT HIGH-ENERGY PHOSPHATE COMPOUND IN TRYPANOSOMATID AND APICOMPLEXAN PARASITES AND A NON-METABOLIZABLE ANALOG

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We have found, using high field 31P nuclear magnetic resonance spectroscopy, that inorganic pyrophosphate (P2O74-) is the major high energy phosphate compound in the parasitic protozoa *Trypanosoma cruzi, Trypanosoma brucei, Leishmania amazonensis,* and *Toxoplasma gondii*, the causative agents of Chagas' disease, African sleeping sickness, leishmaniasis and toxoplasmosis. In all these organisms pyrophosphate is 5-15 times more abundant than ATP; specific enzymatic and chromatographic assays confirmed these results. High-performance liquid chromatographic analysis of perchloric acid extracts of *T. cruzi* epimastigotes labeled for 3 hr with 32P-orthophosphate showed significant incorporation of theprecursor into pyrophosphate, indicating an active metabolic turnover. Subcellular fractionation studies showed that pyrophosphate was not uniformly distributed in *T. cruzi* cells, being mostly associated with acidocalcisomes, pecialized acidic vacuoles present in trypanosomatid and Apicomplexan protozoa which contain the largest intracellular Ca2+ pool in these cells. 31P-NMR, and chemical analysis of the isolated organelles confirmed that they contained large quantities of pyrophosphate, probably in the form of a microcrystalline aggregate. The abundance of this compound, together with the presence of a proton-translocating pyrophosphatase activity in acidocalcisomes suggest that it may fulfill a primary role in the survival of the parasite. This was confirmed by the observation that pamidronate, a pyrophosphate analog currently used in humans in the treatment of bone resorption disorders, blocked the activity of acidocalcisomal pyrophosphatase and selectively inhibited the intracellular proliferation of *T. cruzi* amastigotes and *T. gondii* tachyzoites, with a 50% inhibition at about 65 and 45 µM pamidronate respectively.

RT20 - ADVANCES IN TRYPANOSOMATIDS KNOWLEDGE IN THE LAST 25 YEARS

RT20 - THE CHANGING FIELD OF IMMUNOLOGY IN EXPERIMENTAL LEISHMANIASIS DURING THE LAST TWENTY-FIVE YEARS

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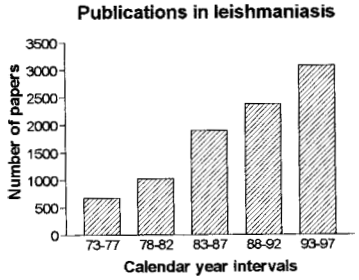
During the last 25 years, there has been a remarkable increase in the publications dealing with leishmania and leishmaniasis. A MEDLINE search (by Internet looking for "leishmania*") revealed 120 papers published in 1973, and 551 papers in 97 (a comparison of '74 to '98 is not yet possible since the search was performed in September 98). These numbers do not represent occasional findings in these specific years, as observed by the steady increase when data is obtained by 5-year intervals:



Of course, several fields experienced a large increase during this same period, but not always as impressive. Just for the sake of comparison, publications about *Trypanosoma cruzi* (a search using the terms "*cruzi* or Chagas disease") revealed 110 papers in 1973 (the number representing 98% of the number of those published in leishmania), and 375 in 1997 (68% of the number observed in leishmania).

Changes observed in interests and approaches in leishmaniasis from 1974 to 1998 largely reflect the introduction of new paradigmas, tools and approaches in the fields of immunology and molecular biology. Themes and interest of today were already present in the 1974 literature (T cell responses, monocytes, vaccines, surface polysacharides), but some of the topics that are similar in title are almost unrecognizable today (cell cooperation but not dealing with cytokines, for example).

Initially without a direct link to immunology, two important contributions in the field of immunology were the demonstration of the differences between log-phase and metacyclic promastigotes and the importance of sand-fly saliva in the establishment of infection. Besides their importance to infection, both findings proved related to the immunological field. Promastigotes from different stages seem to elicit different responses from parasitized macrophages, and macrophage inhibitory effects have been described from products in sand-fly saliva.

Fundamental contributions to our comprehension of leishmaniasis, such as genetic regulation of susceptibility and resistance to infections, were hot topics in the period of 1973 to 1977. Several demonstrations have been published concerning different species of Leishmania. Today's literature expresses a deep interest in the mechanisms of control exerted by molecules identified by genetic screening, such as Nramp-1.

IFN-γ was the first cytokine to receive a prominent attention as a host protective molecule, but has been obliged to share the stage with several other important molecules. Other cytokines which may be involved in lesion development, such as TNF-α or TGF-β received also considerable attention. As for today, IL-12 certainly took from IFN-γ the prima-donna role as the single molecule receiving most attention in leishmaniasis. The roles of IL-12 in driving the response to a protective pole, and its importance as a vaccine adjuvant are highlighted in the literature of the last years of this period.

Models to accommodate similar or competing roles of different cytokines were developed. The comprehension of immuno-regulation in leishmaniasis was strongly marked by the Th1 x Th2 paradigm. Murine models of leishmaniasis were key stones in the consolidation of this model. The opposite roles of IL-4 and IFN-γ, and the predominance of early produced IL-4 in susceptible animals with a corresponding predominance of IFN-γ in resistant animals, fitted nicely in the expected predictions. Several other cytokines have been investigated in this setting, and important aspects of IL-10 have been described. As a matter of fact, the prominent role of IL-4 was difficult to show in human disease, where IL-10 seems to be of great importance in disease development.

Any regulatory mechanism needs effector elements. and the single most important effector molecule for *Leishmania* killing in the last 25 years is certainly nitric oxide (NO). Considerable effort has been applied to contribution of NO and the NO synthases in the elimination of many parasites, including in leishmaniasis. The relevant role of NO demonstrated in the murine models are not paralleled so far by similar findings in the human disease.

The use of knock-out or transgenic mice are important tools for a precise evaluation of the role of cytokines, or NO, as well as of the immune cell repertoire in leishmaniasis in the last five years. Needless to say, none of such elements are represented in the literature of the '70s.

As for the cells involved, the macrophage is sure of maintaining a fundamental interest in leishmaniasis, being as it is the preferential host cell for the parasite. Importance of several intracellular molecular pathways, as well as the importance of surface molecules on the macrophage, such as CD80, CD 86, or CD 40 have been the target of relevant research. NK cells, as important producers of IFN-g early on infection, had their importance recognized in leishmaniasis. Dendritic cells, and their role as *Leishmania* carriers from the injection site to the lymph nodes, and the role of other antigen-presenting cells, such as epidermal Langerhans cells, have also been the subject of considerable interest.

Vaccines were a scarce item in the leishmaniasis literature 25 years ago. Interest in vaccination has largely expanded, since it represents the long-term hope for controlling leishmaniasis. As a large number of antigens among *Leishmania* species are cross-reactive, it is conceivable that a single vaccine with common antigens could induce protective immunity against different forms of disease. A highly effective vaccine against CL remains to be reported, however.

Several interesting approaches to vaccination have been tried. The use of crude antigens of the old literature has been replaced by several promising recombinant antigen candidates. The use of cytokines or cytokine genes in combination with leishmanial antigens in order to enhance immunogenicity is an active field, with strong emphasis on the effect of IL-12. Attempts of vaccination using radioatenuated parasites were popular sometime ago. BCG as protective agent against leishmaniasis , in vitro and in vivo, is a theme that appeared in the '70s. Presently, the use of live, avirulent parasites obtained by gene replacement, or of other microrganisms (such as BCG or *Salmonella*) carrying *Leishmania* genes, receive more attention. In this same line, microrganisms carrying cytokine genes may be useful tools in vaccination, with the desirable perspective of oral administration. Important contributions are being made on the protection obtained by vaccination by the naked DNA approach.

Of course, this text did not cover all the relevant aspects of the immunology of experimental leishmaniasis in the last 25 years. Even when restricting ourselves to murine models, this constitutes a formidable and daunting task. The use of hamsters, dogs, monkeys and other animals have, of course, contributed to our comprehension of several aspects. Even in the mouse models, several aspects were not cited despite their importance. Besides limitations of space and time, we must also recognize our personal bias.

RT20 - IMMUNOLOGY OF *TRYPANOSOMA CRUZI* INFECTION: WHAT IS GOING ON

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Over the past 25 years, research in immunology of experimental and human *Trypanosoma cruzi* infection underwent marked expansion, and has attracted the interest of immunologists beyond the frontiers of the disease's geographical location. The impressive progress in the field is dictated, not only by refined analytical tools, such as genetically engineered mice made available to dissect molecular pathogenesis, but also by structural and molecular biology techniques, allowing resolution and modifications of relevant parasite molecules.

In experimental murine models of infection, a central anti-parasite effector mechanism has been identified as Nitric Oxide (NO) production, driven by iNOS and regulated by the cytokines IFN-g and TNF-a. Mice genetically deficient in the IFN-g receptor or iNOS are highly susceptible to infection (1), while studies in mice deficient in TNF-a also highlighted the role of this cytokine in the immunopathology associated with infection (2). Early priming of the host for increased and continued NO production is assured by a rapid and intense innate type-1 immune response driven by NK cells and DN abTCR T cells (reviewed in 3). Innate immunity however, controls parasite replication only in the first two weeks of infection, as seen in genetically targeted animals, deficient in both T and B cells (4). As infection progresses in the competent host, parasite load is reduced and kept under control in tissues and bloodstream, but the parasite clearly persists. Thus, molecular pathogenesis of persistent infection has been under intense investigation. Perhaps the most relevant lesson from studies of type 1 effector mechanisms against *T. cruzi* is that they are all double-edged swords. Cytokine-driven secretion of NO has been implicated in immunosuppression of T cells through functional arrest (5) and in lymphocyte killing by apoptosis (6). In addition, it has been impossible to dissociate pro-inflammatory (IL-12, IFN-g, TNF-a) from anti-inflammatory (IL-10, TGF-b) cytokine production early in infection. Disrupting the IL-10 gene ammeliorates the control of parasite load, but on the other hand, leads to fatal immunopathology by type 1 cytokines (7). The parasite appears to be spared amid this insoluble conflict of the host. Perhaps the most crucial event at this stage, is the extinction of classical Th1 T-cell responses, as measured by IL-2 production (8). Activation-induced cell death (AICD), an intrinsic and regulatory response of Th1 T cells, has been described in *T. cruzi* infection (9), and was suggested as a parasite-driven mechanism for extinguishing Th1 T cell responses (3,10). AICD is mediated by CD95-CD95L interactions, and exacerbates *T. cruzi* growth in infected macrophages (10). Surprisingly, CD95L-mutant (*gld*) BALB mice deficient in AICD, mount immune responses markedly deviated to Th2-type (IL-4, IL-10) upon infection, which render them more susceptible than controls (this meeting). Since lymphocyte apoptosis also occurs *in vivo* in infection (6,9), it could

have direct pathogenic effects, given the recent descriptions of an anti-inflammatory (deactivating) action of apoptotic bodies on macrophages. In fact, phagocytosis of apoptotic lymphocytes increases *T. cruzi* replication in macrophages, and injection of apoptotic cells into infected mice exacerbates parasitemia (this meeting).

Research on molecular pathogenesis must be based on identification and structural characterization of parasite molecules responsible for altered host responses, such as lymphocyte activation, apoptosis and suppression. Some molecules with immunoregulatory activity were either cloned or identified in *T. cruzi* and, in some cases, their effects on host cytokine and cellular responses were investigated. GPI-anchored parasite surface glycoconjugates comprise an important group of bioactive molecules. Among them, mucin-like glycoproteins from trypomastigotes induce pro-inflammatory cytokine and NO production in IFN-g-treated macrophages (11), while an epimastigote mucin induces IL-10, but not IL-12 production (12). The major glycoinositolphospholipid (GIPL) from *T. cruzi* suppresses naive T-cell activation through its ceramide lipid domain (13), induces polyclonal Ig synthesis by B-cells (14), and synergizes with IFN-g to induce macrophage apoptosis (15). A parasite-released protein factor (TIF) acts on normal human T cells to block IL-2 production and expression of some selected surface antigens, including IL-2 Receptor chains, involved in T-cell activation (16). A parasite-released protein named Tc52, homologous to glutathione S-transferases, synergizes with IFN-g to increase iNOS expression and NO production in macrophages, while downregulating IL-10 expression (17). This protein also suppresses T-cell responses by a mechanism of glutathione depletion, that could be similar to certain effects of NO on T cells (17). Another important molecule is the GPI-anchored and shed parasitic enzyme *Trans*-sialidase, which has been identified as a potent determinant of virulence in *T. cruzi*-infected mice (18). The mechanisms of action of *Trans*-sialidase in the host immune system are now under investigation (this meeting), and it appears that the enzyme is able to exert effects on lymphocytes that could be relevant for immunopathology and autoimmunity.

The goal of immunoprotection against *T. cruzi* infection has eluded researchers, in spite of great effort and recent progress towards immunization with recombinant proteins and recombinant DNA (this meeting). Both CD4+ and CD8+ T-cell subsets are required for host protection against *T. cruzi* (19,20). Besides lytic antibodies (21), cytotoxic responses mediated by CD8+ T cells are thought to be essential effector mechanisms for host protection. This notion is based on the cytosolic location of the parasite, which favors MHC class I antigen display, and also on the fact that many infected cells do not express class II molecules. The interest in cytotoxic responses is further justified by the recent finding that infected macrophages, while defective for presentation of antigen via MHC class II, are able to present antigen via MHC class I in a normal fashion (22). Studies with genetically engineered parasites indicate that both secreted and GPI-anchored parasite proteins would enter the host MHC class I pathway when the parasite infects a target cell (23). A screening of *T. cruzi* proteins, identified a Tc85-derived peptide that binds to MHC class I and is recognized by cytotoxic CD8+ T cells from naturally infected mice (24). Moreover, a T-cell line reactive with this peptide was able to confer protection against *T. cruzi* when transferred to recipient mice (24).

The recent findings, both mentioned herein and presented at this meeting, illustrate the point that research in immunology of Chagas'disease has gone a long way towards understanding molecular pathogenesis and immunoprotection. It has also become a respected and prominent area of host defense immunology. However, the whole story is yet to be told, perhaps in a few years ahead.

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RT20 - *TRYPANOSOMA CRUZI*: AND CHAGAS' DISEASE: A CRITICAL ANALYSIS OF RESULTS OBTAINED IN THE LAST 25 YEARS

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The 1970s and 1980s were characterized by a series of advances, mainly in the immunology and immunopathology of Chagas' disease, as well as in the biochemistry, ultraestructure and interaction of *Trypanosoma cruzi* with the host's cells, summarised by by Brener (1973, 1980), with excellent reviews on the biology abd immunology of the o parasite. A complete reexamination of Chagas' disease, from the parasite to prophylaxis, was undertaken by sixteen Brazilian authors and published in a book "*Trypanosoma cruzi* and Chagas' disease, edited by Brener and Andrade (1979).

Of particular note among recent advances in the work by Cossio et al. (1974) and Szarfman et al. (1977) on EVI antibodies. Szarfman et al. (1982) subsequently showed that they did react specifically with *T. cruzi*, but instead connective tissue structures, mainly laminin. Teixeira and Santos-Buch (1975) carried out pionneeering experiments on rabbits showing that delayed hypersensitivity mechanisms through lymphocytes sensitised with *T. cruzi* can produce lesions similar to those of chronic Chagas' disease in human. The characterisation of *T. cruzi* surface glycoproteins by Snary and Hudson (1979) and Snary (1980, 1985) was pf enormous importance due to its potential as a specific diagnostic test as well for producing a Chagas' disease vaccine. Various other related aspects have been recently reexamined by Nogueira and Coura (1990). Along the same lines, Brener and Krettli have made a critical study of progress in Chagas' disease immunology, including the defence mechanisms of the host, immunopathology and the outlook for a vaccine.

From the standapoint of serological diagnosis the development of hemaglutination and ELISA assays for Chagas' disease by Neal and Miles (1970) and Voller et al. (1975) respectively, have been significant advances. On the other hand the description of the so-called "lithic antibodies" by Krettli and Brener (1982) was an important step for serologic diagnosis mainly in the parasitological cure control.

The use of biochemical approach to group *T. cruzi* strains from animals and humans, as by isoenzyme (Zymodeme) analysis carried out by Miles et al. (1977), or by the kinetoplast DNA studies using cleavage with restrictive endonuclease (Schizodemes), as introduced by Mattei et al. (1977), and by Morel et al. (1980), constitute one the useful tools of recent years not only for the verification on intra-and inter-specific variations of *T. cruzi*, but also because of their value in epidemiological studies and diagnostic technique.

Mechanisms for *T. cruzi* interaction, penetration and escape in cells pertaining to the host immune system, studied by Dvorak and Schmunis (1972), Dvorak and Hyde (1973), Nogueira and Cohn (1976), Krettli and Eisen (1980), Nogueira et al. (1980) as well as the action of drugs and the death of the intracellular parasite, investigated by Do Campo et al. (1981), stand as exramely important milestones in our understanding of the biology and pathology of hos parasite-cell interaction.

New light has bee thrown on the pathogenesis of Chagas' disease by studies of the ultrastructure of muscular, interstitial and nerve components of the heart, esophagus and intestine in experimental and human forms of the disease, carried out by Tafuri (1974) during the 1970s, and the improved canine experimental model by Andrade and Andrade (1980) and by Andrade et al. (1980, 1984, 1987).

Further knowledg of the triatominae vectors of Chagas' disease, their biology and geographical distribution were summarised by Sherlock (1979), and by Lent and Wygodzinsky (1979). Three aspects are particularly noteworthy: studies by Forattini (1980), on the biobeography, origin and distribution of triatominae in Brazil; by Zeledon (1983), on the ecophysiological characteristics of Chagas' disease vectors, and by Perlawagora-Szumlewicz (1982,1987,1988) on xenodiagnosis and the *T. cruzi*-triatominae interrelationship.

Important work in the field of epidemiology, morbidity and control during recent decades includes studies by Macedo (1973) Lopes et al. (1975), Dias (1982, 1987), Prata and Macedo (1984),Coura (1975,1988), Coura et al. (1983, 1984, 1985), Coura and Pereira (1984) and Pereira et al. (1985), that reveal the natural history of Chagas' disease of which little was previously known.

Deane et al. (1984, 1986), supplemented by Lenzi at al. (1984) discovered a double *T. cruzi* cycle in the anal glands of the opossum *Didelphis marsupialis.* These glands showed developmental stages of the parasite usually found only in vertebrates or in invertebrate host. This finding will revolutionize the established concepts of parasite life cycle and the epidemiology of the disease. On the other hand the epidemiology of Chagas'disease in the Brazilian Amazon as an anthropozoonosis has been recently emphasized by Coura et al. (1994a,b, 1995a,b).

From the end of the 1980s to 1995 a series of advances were made in the area of diagnosis of Chagas'disease by molecular techniques, using recombinant antigens for serodiagnosis, as summarized by Silveira (1992) , and parasite detection in chronic chagasic patients by PCR amplification of kinetoplast DNA, as described by Sturm et al. (1989), Avila et al. (1991) and Britto et al. (1993, 1995).

We know a great deal, yet we know so little about Chagas' disease! Berenice, the child involved when Chagas first described the acute phase of the disease in 1909, lived for another 70 years (Salgado 1980) with *T. cruzi* circulating in her blood, but showed no sumptoms or signs of the chronic phase of the disease!

RT20 - CELL BIOLOGY OF TRYPANOSOMATIDS: A SHORT SURVEY OF THE PRESENT SITUATION AND NEW PERSPECTIVES

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In the last 25 years we observed a dramatic increase in the number of colleagues working with protozoa of the Trypanosomatidae family applying a variety of cell biology techniques to examine basic questions related with the biology of these eukaryotic microorganisms. It is almost impossible to summarize all data in a few pages. Therefore, I decided to point out some major contributions in some specific areas, as well as to indicate some not yet solved questions which, in my opinion, deserve further investigation in the coming years.

The Protozoa

The Cell Surface - Nothing was known on this topic twenty five years ago. During this period information were obtained on the (a) presence of surface-exposed glycoconjugates, some of which have been isolated and characterized, forming a glycocalix, (b) identification of a novel mechanism of sialylation of glycoconjugates in *Trypanosoma cruzi* via a *trans*-sialidase, interfering with important phenomena such as interaction with host cells, complement activation, etc, (c) determination of the surface charge and analysis of its role on the process of *T. cruzi*-host cell interaction, (d) verification that several macromolecules are associated with the plasma membrane through a GPI anchor, (e) verification that components of the plasma membrane move in the membrane plane, with processes of patching and capping and subsequent shedding of small vesicles into the extracellular medium, etc. In the last years some attempts were done trying to establish relationships between surface exposed components, identified using antibodies, and intramembranous components revealed in freeze-fracture replicas. Some evidence have been obtained showing marked differences in the structural organization, composition and fluidity of the plasma membrane of the various developmental stages of the parasite's life cycle. Despite all new information obtained there are several important questions to be answered. For instance, it will be important to define the three-dimensional organization of key membrane associated glycoproteins such as members of the *trans* sialidase family. Although there are indications that the fluidity of the plasma membrane varies according to the developmental stage no quantitative data on the membrane fluidity have been reported. The modulation of the insertion of macromolecules in the membrane in response to external stimuli or intracellular changes has not been analyzed. Even the advantage of the existence of a large number of macromolecules associated with the plasma membrane via a GPI anchor has not been analyzed in detail.

The cytoskeleton - We significantly increased the knowledge on the cytoskeleton of trypanosomatids, especially from the morphological point of view. Filamentous bridges connecting the sub-pellicular microtubules to each other, with the plasma membrane and with profiles of the endoplasmic reticulum, have been described. However, the proteins which make the bridges were not identified and characterized. High molecular weight proteins, some of which are highly antigenic, have been identified at the region of adhesion of the flagellum to the cell body. Freeze-fracture studies have revealed the existence of a specialized cell junction at this area. However, the junctional proteins were not identified. One interesting observation was that showing detachment of the flagellum from the cell body in epimastigotes of *T. cruzi* following knock out of the gene coding for Gp72, a glycoprotein first shown to be involved in binding to complement components. Actin and actin-associated proteins have been found in trypanosomatids using immunological techniques. However, actin filaments were not observed. One special cytoskeletal structure of trypanosomatids is the paraflagellar rod, made by a complex array of filaments of various types. A three-dimensional model for this complex structure has been proposed and two major proteins have been identified. Other important proteins will be identified using a recently obtained pure fraction containing this structure. Recent experiments with mutants of *T. brucei* and *C. fasciculata* which do not express one of the genes coding for one of the major proteins suggested that the paraflagellar structure play some role on the flagellar movement.

We know very little about the mechanisms involved in the control of the organization of the cytoskeleton and the changes which take place during the process of differentiation (or transformation), which involves accentuated modifications in the shape of the protozoan.

The endocytic pathway - Important information were obtained on the mechanisms used by trypanosomatids to incorporate macromolecules from the extracellular medium, especially in *T. brucei* and *T. cruzi*. Both protozoa ingest macromolecules through a receptor-mediated (LDL and transferrin) endocytosis as well as by adsortive endocytosis. In most of the trypanosomatids this process takes place in the flagellar pocket. In the case of epimastigotes of *T. cruzi* most of the endocytic vesicles are formed in the cytostome, a specialized region of the cell surface which has been characterized using freeze-fracture and cytochemistry. The ingested macromolecules are concentrated in a slightly acidic organelle, known as reservosome, which gradually disappears during transformation of epimastigotes into trypomastigotes. The study in this area is at the beginning and certainly new information will be obtained after isolation of the organelles involved in the endocytic pathway, and the identification of protein markers such as rabs, as described in mammalian cells. The endocytic pathway in *Leishmania* has not been characterized. Some attempts to induce ingested of gold-labeled proteins by promastigotes failed. However, intravacuolar amastigotes were able

to ingested gold-labeled transferrin discharged into the parasitophorous vacuole following fusion of endosomes/ lysosomes. In amastigotes of some *Leishmania* species there is a large lysosome, known as megasome, which concentrates cysteine proteinase.

The glycosomes - One important advance was the observation that trypanosomatids present a special peroxisome which concentrates enzymes of the glycolytic pathway. This organelle has been found in all trypanosomatids, varying in number according to the growth conditions. Their number is reduced when a oxidative metabolism is activated, increasing when cells are grown in media rich in sugars as carbon sources. New information have been obtained with the identification of signal sequences for the targeting of proteins synthesized in the cytoplasm and then incorporated into the glycosomes. Peroxisomes of mammalian cells perform additional functions, such as b oxidation of lipids. Studies are necessary to check for additional functions of the glycosomes.

The acidocalcisome - The presence of electron dense inclusions in the cytoplasm of trypanosomatids has been reported since the early observations of thin sections by transmission electron microscopy. Evidence were obtained in the last years that this structure is acidic and involved in the uptake of calcium, being designated as acidocalcisome. The membrane of the organelle presents a vacuolar-type proton ATPase and a calcium-ATPase. The number of acidocalcisomes varies among the trypanosomatids and according to the developmental stage. Further investigation are necessary to clarify the biogenesis of this organelle, targeting mechanisms for insertion of its macromolecules, role on the regulation of calcium uptake and concentration within the organelle.

The kinetoplast - A large amount of information was obtained about the kinetoplast, a rod-like structure with a mean length of 1 mm and a thickness of 0.1 mm, which presents a high concentration of extranuclear DNA (up to 30% of total DNA). Electron microscopy have shown that it consists of a network of 20000 to 30000 associated minicircles which are maintained in shape probably due to the presence of proteins. The minicircles are involved in the formation of small guide RNAs which control the specificity of the process of mRNA editing in the organelle. Maxicircles, similar to those found in mitochondria of other cells and which encode rRNAs and mitochondrial proteins involved in electron transport and ATP synthesis, are also observed. One characteristic feature of maxicircle transcripts is the process of RNA editing, a process by which uridine residues are inserted at the internal sites within the sequences. The process of replication of the kinetoplast DNA has been the subject of intense investigation in the last few years. During this process the number of minicircles and maxicircles must double. It was shown that in order to minicircle replication occurs it is released by a topoisomerase II, replicating as a free minicircle. Later on the minicircle is re-attached at antipodal positions on the network periphery.

It has been suggested that the kinetoplast disk actually spins during replication.

Parasite-Host Cell Interaction

Cell recognition - A large amount of information has been obtained about the initial contact between *T. cruzi* and *Leishmania* with the host cells. It is now clear that glycoconjugates exposed on the parasite surface play an important role on this process. In the case of trypomastigote forms of *T. cruzi* glycoproteins of 35/50 kDa (metacyclic) and 70-90 kDa (tissue culture derived) seem to be relevant to the interaction process. These proteins can be sialylated in a process in which the parasite trans-sialidase is involved. Some of them, like Tc-85 and penetrin, are able to interact with extracellular matrix components such as fibronectin, laminin, collagen and heparan sulphate. The sialic acid residues can be removed by the same enzyme acting as a neuraminidase. In the case of promastigotes of *Leishmania* a lipophosphoglycan and Gp63 are involved in the interaction with macrophages. From the published results it is clear that several surface-exposed molecules play some role on the process of parasite-host cell interaction. It is possible that the parasite indeed presents several alternative possibilities to perform a vital function. However, we can not exclude the possibility that the molecules are involved in different steps of the interaction process, such as (a) initial recognition, (b) cell signaling, and (c) invasion. It is expected that new studies will determine the role of each molecule in these different steps which have been usually assayed as only one step.

In the case of *T. cruzi* there are very few information on the nature of the components of the host cell surface involved in the interaction process. However, there are evidences that surface glycoconjugates, including those containing sialic acid, are important. In the case of *Leishmania* the involvement of macrophage receptors for complement and mannose has been demonstrated.

Invasion - The available data indicate that once attached to the host cell surface *T. cruzi* triggers cell signaling processes involving protein phosphorylation and in some cell types, calcium release (both in the parasite and the host cell) and actin polymerization. In some cells migration of lysosomes towards the region of parasite invasion is observed. Concomitant with this process there is the formation of a transient parasitophorous vacuole, with participation of the plasma membrane of the host cell and the membrane of lysosomes. There are few information on the biogenesis of the parasitophorous vacuole and the composition of its membrane. Protein phosphorylation is also involved in the process of infection of macrophages by promastigote forms of *Leishmania*. However, protein tyrosine kinase antagonists that inhibit macrophage infection by promastigotes do no interfere with infection by amastigotes.

Intracellular differentiation (transformation) - It is well established that a few hours after invasion the trypomastigote form of *T. cruzi* and the promastigote form of *Leishmania* transform into a rounded amastigote form. There are no data on the nature of the signals which induce such transformation. In the case of *T. cruzi* the amastigote form remains quiescent for many hours (24 hs in some strains) before start a process of cell division. After several division cycles the amastigote forms transform into the trypomastigote forms. Again there are no information about the nature of the signals which induce such transformation.

Molecular basis of the tropism - The question of the tropism presented by some strains of *T. cruzi* is not clarified from a molecular point of view. Now it is possible to re-analyze this question using well characterized surface molecules (exposed CAMs, Cadherins, Integrins and Selectins) of the host cells.

Life Cycle in the Insect

Despite its importance in the life cycle there are few new data on the interaction of *T. cruzi* and *Leishmania* with the invertebrate host. There are evidences that surface-exposed LPPG and LPG from the parasites are involved. However, it is not clear if these molecules bind to specific receptors present in the epithelial cells or in the peritrophic and perimicrovillar membranes of the gut. Certainly cell signalling mechanisms also operate in the invertebrate host and this area has been neglected. However, it has been shown that a peptide derived from the globin is able to activate *T. cruzi* adenyl cyclase inducing the process of epimastigote-trypomastigote transformation.

RT20 - TWENTY FIVE YEARS OF PARASITE BIOCHEMISTRY IN CAXAMBU

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Twenty seven years ago, while enrolled in a Course on Yeast Genetics at Cold Spring Harbor I had the courage to ask a Nobel Prize winner whether he knew anything about viruses that infected protozoans. I had just returned to Brazil from my post-doctoral training in New York and was trying to write a project on protozoan biochemistry selecting initially *Trypanosoma cruzi* and *Acanthamoeba castellanii*. There were at least 55,000 described species of protozoans and they were so ill studied, particularly at the molecular level. Additionally, protozoans were a cause of much suffering and disease in developing countries. At the time I had vague ideas on which approaches to pursue and wanted opinions. The Nobel Prize winner stared at me and referring to the protozoans answered with a question: "Of what use are they?". Of course, he was manifesting a prejudice very common to scientists who, from time to time, elect the fashionable systems to study. At that time they were rat liver mitochondria, yeast and *Escherichia coli* (with or without l phage).

In spite of this, these past 27 years served to demonstrate that protozoans can be instruments of original discovery in Biology, and the Caxambu meetings cooperated in catalysing a general interest in molecular aspects of these organisms, particularly *T. cruzi* and *Leishmania*. It is my opinion that this meeting generated a focus of interest which spread to other South American countries and soon, scientists, mainly from Argentina, Venezuela and Chile, joined to the general efforts made to study biochemical aspects of these parasites. As a matter of fact, a network of collaborations has been established in South America in the areas of Chagas' disease and Leishmaniasis. Of course many important contributions have been made elsewhere, but the Caxambu meetings not only became a resonance box of the best research done in the world in those areas but was also an instrument to attract attention of the Southern Cone authorities to the necessity, at least, of Chagas' disease erradication. These efforts have been efficiently complemented by the concerted actions of clinicians and epidemiologists and the intervention of the World Health Organization.

Thus, it appears that parasites indeed served some purpose since they gave us the notions of RNA editing, trans-splicing, antigenic variation, trypanothione involvement in the redox metabolism and are presently being used as tools to the understanding of host cell recognition and intracellular signaling. However, most relevant to our anniversary was the heavy participation of South American scientists (and Caxambu was the forum) in, for example, the description of the arginine-ornitine cycle in these parasites, the specific proteases which might be important in *T. cruzi* invasion of cells (as it appears to be the case with *Leishmania*), and the mucins and their molecular organization. Important also were the discoveries of the transient glycosylation during protein processing and its involvement in the establishment of the correct protein conformation, the glycosylphosphoinositol glycerides and ceramides, a novel class of molecules which somehow paved the way for the disclosure of the anchor structures, the sialyltransglycosylase (sialidase), and the hemoglobin derived peptide which seems to control via signal transduction and adenylyl cyclase the differentiation of *T. cruzi* epimastigotes. Practical approaches, which resulted from fundamental research have also been attempted as, for example, the development of diagnostic kits based on specific configuration of sugars and on recombinant proteins.

I have been told by the organizers to restrain myself to Biochemistry but nowadays it is difficult to separate that branch of Biology (or Chemistry?) from Cell Biology, Molecular Biology, Chemotherapy and Chemistry. In that sense, I will finish this abstract trying to guess which applications might be important in the next decade, slightly mixing these rapidly fusing disciplines. First of all, it must be said that the unraveling of small and medium sized genomes, due to its speed, is becoming an ever growing strategy to disclose the metabolic circuitry of a given organism. Thus, it can be expected that the genomes of *T. cruzi* and *Leishmania* will soon be completely sequenced and we will consequently be in a position to learn more about problems which, as yet, could not be efficiently approached by the scientists. These problems deal on the one hand with the understanding of the molecular basis of parasitism and on the other, with the discovery of drugs for efficient treatment of the diseases. These are: (1) the biochemical processes which permit *T. cruzi* and *Leishmania* to live inside cells or, in other words, the biochemistry of the intimate host-parasite relationship which is the essence of parasitism; (2) the signals that trigger differentia-

tion and the differential expression of genes at each parasite stage, allowing the understanding of the fine metabolic imbalance presiding the strategy of parasitism; (3) the chemical mechanisms involved in parasite interaction with cells of the immune system and the role of the major surface molecules in these processes, essential to the comprehension of the immunopathology of the diseases; (4) certain specific metabolic pathways as those related to the transport of nutrients across the membrane or leading to GIPLs and glycoprotein biosynthesis, for example; and (5) the disclosure of three-dimensional structures from several specific enzymes, e.g. enzymes of the trypanothione metabolism, of the galactofuranose metabolism – important carbohydrate belonging to major membrane structures like LPPG, GIPLs, mucins, and LPG – or from any other enzyme with special characteristics which differentiate it from its vertebrate hosts counterparts, thus allowing the design of drugs for rational therapy.

Of course, as usual in science, all these guesses may prove to be wrong in face of unpredicted new discoveries that may change the whole course of events.

RT20 - CHEMOTHERAPY OF CHAGAS' DISEASE AND LEISHMANIASIS: THE LAST 25 YEARS

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The last quarter century have witnessed tremendous advances in the understanding of the biochemistry and physiology of *Trypanosoma cruzi* and several species of the genus *Leishmania*, as well as in the studies of the cellular and molecular basis of their interactions with their vertebrate hosts. Although these advances have not translated yet in new chemotherapeutic resources for the treatment of the diseases caused by these organisms, the accumulated knowledge have led to a new wave of developments which could result in important breakthroughs in the near future. In this brief review I will discuss the most important advances in research and development relevant to the chemotherapy of Chagas' disease and the several types of Leishmaniasis, with particular emphasis in those developments that have completed pre-clinical studies or have reached clinical trials.

Chagas' disease

Nitrofurans and Nitroimidazoles - The nitrofuran derivative nifurtimox (Lampit®, Bayer) and the nitroimidazole benznidazole (Rochagan®, Roche) were empirically introduced over 25 years ago for the treatment of Chagas disease. In the course of this time period numerous studies have shown that both compounds have significant anti-*T.cruzi* activity in human acute infections and those with a relatively short development period (months to a few years) [1-5]. However, the percent of therapeutic efficacy varies geratly among different geographical areas, probably due to differences in the intrinsic susceptibility of *T.cruzi* strains throughout the endemic areas [1,2]. However, these compounds have repeatedly been shown to have very limited or no efficacy in the treatment of the prevalent chronic form of the disease [2,3,5]. However, despite the fact that parasitological cures cannot be achieved in most cases, longitudinal studies have shown that benznidazole treatment can slow the progression of chronic form of the disease [6]. On the other hand, both drugs have frequent side effects (generally less severe in pediatric patients) including anorexia, vomiting, peripheral neuropathy and allergic dermopathy [2,3,5]. Basic studies in the last two decades have helped to illuminate the molecular basis of both the anti-*T.cruzi* activity and toxicity of these compounds [7,8]. Nifurtimox acts via the reduction of the nitro group to very reactive nitroanion radicals, which in turn react to produce highly toxic reduced oxygen metabolites. *T.cruzi* has been shown to be deficient in detoxification mechanisms for oxygen metabolites and is thus more sensitive to free radicals than vertebrate cells. Benznidazole seems to act via a different mechanism (reductive stress), which involves covalent modification of macromolecules by nitroreduction intermediates. Nifurtimox production has been recently discontinued.

Allopurinol - This pyrazolopyrimidine has been used for a long time in humans for the treatment of gout, as it is transforme in vertebrates into oxypurinol, a potent inhibitor of xanthine oxidase. In trypanosomatids, which are deficient in xanthine oxidase, the compound acts as a purine analog as in incorporated into nucleic acids, disrupting the synthesis of RNA and proteins [3]. The compound was shown to be active in murine models of acute Chagas disease, but marked differences in susceptibilities among*T.cruzi* strains were also reported [9,10]. There have been conflicting reports of the therapeutic efficacy of this compound in humans [1], but a multicentric study launched in 1992 in Argentina, Brazil and Bolivia was stopped as there were relapses of parasitemia after three months [11]. The results of the Bolivian study, released in 1994, showed that allopurinol had only suppressive activity as 92 % of chronic patients treated with 900 mg per day for 60 days had positive xenodiagnostic results one year after treatment [12].

Sterol biosynthesis inhibitors - T.cruzi requires of specific endogenous sterols for cell viability and proliferation and is extremely sensitive to sterol biosynthesis inhibitors (SBI) in vitro [13-19]. However, currently available SBI's, which are highly successful in the treatment of fungal diseases [20-22] are not powerful enough to eradicate *T.cruzi* from experimentally infected animals or human patients [23-25]. We have recently demonstrated that D0870 (Zeneca Pharmaceuticals), a bis-triazole derivative which is the R(+) enantiomer of ICI 195,739 [16,26-28] is capable of inducing parasitological cure of both acute and chronic experimental Chagas disease [19,29], the first compound ever to display such activity. More recent work have demonstrated that SCH 56592, another triazole

derivative currently undergoing clinical trials as a systemic antifungal, has anti-*T.cruzi* activity comparable or superior to that of D0870 [30]. Both compounds are active *in vivo* against *T.cruzi* strains partially or highly resistant to nifurtimox and benznidazole [31]. The special anti-*T.cruzi* activity of these fourth-generation azole derivatives has been attributed to both their selective action on their parasite target (cytochrome P-450 dependent sterol C14 demethylase) and their special pharmacokinetic properties [19,29,30]. Although development of D0870 has recently been discontinued, SCH 56592 is a logical candidate for clinical trials with chronic Chagas disease patients.

Other biochemical aspects of *T.cruzi* which have been characterized in this period and could potentially be exploited as chemotherapeutic targets include: the location of most glycolytic enzymes in a peroxisome-like organelle, the glycosome [32,33]; the presence of a constitutive phospho-*enol* –pyruvate carboxykinase which allows the use of both carbohydrates and aminoacids as carbon and energy sources, through a modification of the Krebs' cycle [32,34-36]; the presence of trypanothione and trypanothione reductase, which replace glutathione and glutathione reductase in trypanosomatids as the main intracellular reducing system [37] and the presence of a cathepsin-L-like cysteine proteinase (cruzipain) which seems to be essential for parasite-host interaction and intracellular development [38-40]. However, up to this date only inhibitors of cruzipain have been shown to have specific anti-*T.cruzi* activity in vitro [41-43] but no in vivo studies have been reported.

Leishmaniasis

Pentavalent antimonials - Despite the impressive advances of the last 25 years in the understanding of the biochemistry and molecular biology of the parasites belonging to the *Leishmania* genus, the mainstay in the treatment of all forms of leishmaniasis are still pentavalent antimonials, which were introduced empirically over 50 years ago [44]. Both sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®) are active in most cases. However, the accompanying side effects, which often result in the interruption of treatment, and the increasing resistance to this type of drugs in many parts of the world clearly indicate the need for a more effective and safer chemotherapeutic approach. Only recently some detailed understanding of the chemical structure and the possible mechanisms of action of these drugs have begun to emerge [45-47]; the limited knowledge on the structure and mechanism of action of these compounds limits our current understanding of drug resistance [44].

New Amphotericin B formulations - The polyene antibiotics, particularly Amphotericin B, have been used for decades in the treatment of fungal infections, as they associate preferentially with ergosterol-containing plasma membranes and disrupt their selective ionic permeability. *Leishmania* parasites are also extremely sensitive to Amphotericin B, as they contain ergosterol-like sterols as the main neutral lipids of their plasma membranes [19,48-54]. However the serious side effects of the conventional formulation of Amphotericin B have limited its use as anti-leishmanial agent [44]. New lipid formulations of this antibiotic in which it is complexed with phospholipids and/or sterols, instead of deoxycholate, have significantly improved therapeutic ratios [55]. They have been successfully used in the treatment of visceral leishmaniasis, including antimony-resistant cases in the Middle East, India and Brazil [56-58].

Sterol biosynthesis inhibitors - As in *T. cruzi*, all parasites of the genus *Leishmania* require specific endogenous sterols for cell proliferation and viability and thus are very susceptible to the antiproliferative effects of sterol biosynthesis inhibitors in vitro [19,48-51,53,54,59]. However the effects of azole derivatives such as ketoconazole and itraconazole on human infections have been equivocal, ranging from high efficacy against *L. mexicana* and *L. tropica* infections to little or no activity against *L. donovani* or *L. braziliensis* [60-69]. Although some of this variability could be attributed to the pharmacokinetic profile of the drugs, recent studies present convincing evidence that it could come from the different intrinsic susceptibilities of the etiological agents [62]. We have recently shown that some natural *L. braziliensis* populations proliferate normally even when all their natural sterols have been replaced with 14-methyl analogs, a fact that poses a fundamental limitation for sterol C-14 demethylase inhibitors as antiproliferative agents against such organisms [54]; however, it was also shown that these organisms are rendered extremely susceptible to azole derivatives when treated simultaneously with the allylamine terbinafine, which block sterol biosynthesis at the level of squalene epoxidase [22,54]. This suggests that combination therapies with sterol biosynthesis inhibitors could be effective in the treatment of azole-resistant *Leishmania* infections.

Allopurinol - Again as in *T. cruzi*, this hypoxanthine analog is incorporated in *Leishmania* cells via the purine salvage pathway, leading to abnormal nucleotides which affect nucleic acid synthesis and expression [44]. However, the results of clinical trials with this compound in both visceral and cutaneous leishmaniasis have been, in general, disappointing [44].

Paromomycin - This aminoglycoside antibiotic has specific and potent anti-*Leishmania* activity but its use has been limited by its poor water solubility, which leads to almost nil oral bioavailability [44]. However, both ointment [70] and injectable [71] formulations have been developed in the last decade and have been used succesfully in the treatment of cutaneous and visceral leishmaniasis, respectively.

Alkyl-lysophospholipids - These phospholipid analogs have cytotoxic a activity against cells with high proliferation rates and are currently being developed as anti-tumor drugs, with good safety profile [44]. They have also been found very active in several animal model of both visceral and cutaneous leishmaniasis [72,73].

In conclusion, it is clear that, despite the long strides made in the study of the basic biology of these two important human pathogens, large gaps remain in our understanding of the molecular basis for survival and pathogenesis in their vertebrate hosts. This clearly limits our capacity to rationally develop new chemotherapeutic approaches. Nevertheless, the identification of specific lipid and protein requirements, particularly at the level the

plasma membranes, for growth and survival is leading to new developments which could culminate in novel chemotherapeutic approaches in the near future.

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GENERATION OF ANTIBODIES WITH FUNCTIONAL AUTOREACTIVE PROPERTIES IN CHAGAS DISEASE

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Anti-receptor antibodies in Chagas disease are a consequence of the recognition of parasite antigens

In Chagas' heart disease, antibodies to the β1, β2 and M2 acetylcholine receptor have been detected (1,2,3). They are directed against their second extracellular loop (1,2,3). Using peptides encompassing this region of each receptor as the immunological basis of an ELISA, a significant increase of an anti-peptide response could be shown in chagasic patients (n=66) compared to that in healthy controls (n=34). Prevalence of the anti-β1, the anti-β2 and the anti-M2 response in controls was 2,9%, while in patients it was 53% for the β1 peptide ($p<0,0001$), 39,4% for the β2 peptide ($p<0,0003$) and 93,9% for the M2 peptide ($p<0,0001$) (3). A similar result was obtained with the corresponding IgG fractions (3). Comparison of the sequence of the C-terminal part of the 34 kDa ribosomal P0 protein from *Trypanosoma cruzi* and the second extracellular loop of the human β1-adrenergic receptor and the M2 acetylcholine receptor showed that they contain a pentapeptide with very high homology: AESEE derived from the P0 protein of *T.cruzi* (peptide P0-β), AESDE in β1 and EDGEC in M2 (2,3). Different studies showed cross-inhibition of the recognition of the peptides encompassing the second extracellular loops from both receptors and the P0-β peptide (2,3,4). Furthermore, it was shown that the agonist like functional activity of anti-β1 and anti-M2 antibodies could be blocked by peptide P0-β (2,3,4). These results led to the hypothesis that this parasite protein could mediate the induction of a functional immune response against the these receptors.

A more direct evidence of the involvement of ribosomal P proteins of *T.cruzi* in the generation of functional autoreactivity against heart tissue has been recently obtained studying the antibody response against the 11 kDa P1 and P2 proteins (5). Antibodies against these proteins are known as anti-P antibodies (measured as anti-R13 antibodies). They are present in sera from patients with chronic Chagas heart disease (cChHD) and recognize the peptide R13, EEEDDDMGFGLFD, which encompasses the carboxy-terminal region of the 11 kDa P1 and P2 proteins. This peptide shares homology with the C-terminal region (peptide H13 EESDDDMGFGLFD) of the human ribosomal P proteins, which is in turn the target of anti-P autoantibodies in systemic lupus erythematosus (SLE), and with the acidic epitope, AESDE, of the second extracellular loop of the β1 adrenergic receptor. Anti-P antibodies from chagasic patients showed a marked preference for recombinant parasite ribosomal P proteins and peptides, whereas anti-P autoantibodies from SLE reacted with human and parasite ribosomal P proteins and pep-

tides to the same extent. A semi quantitative estimation of the binding of cChHD anti-P antibodies to R13 and H13 using biosensor technology indicated that the average affinity constant was about 5 times higher for R13 than for H13 (5). Competitive enzyme immunoassays demonstrated that cChHD anti-P antibodies bind to the acidic portions of peptide H13, as well as to the peptide that encompasses the second extracellular loop of the b1 adrenoceptor (5). Anti-P antibodies isolated from cChHD patients exert a positive chronotropic effect in vitro on cardiomyocytes from neonatal rats, which resembles closely that of anti-b1 receptor antibodies isolated from the same patient. In contrast, SLE anti-P autoantibodies have no functional effect. These results indicate that only the anti-P specificity generated as a response to the parasite P proteins has adrenergic stimulating activity (5).

The anti-P reaction is a hallmark of cChHD

The R13 peptide is not recognized by sera from patients with malaria or leishmaniasis. Moreover, anti-P antibodies are not detectable in sera from individuals infected by *Trypanosoma brucei*, a parasite that contains ribosomal P proteins with the same C-terminal end as P proteins in *T.cruzi*, nor in acute Chagas patients, or in sera from patients with digestive forms of Chagas disease (6). These observations imply that in cChHD, parasite ribosomes are directly exposed to the immunological system. Since in cChHD severe inflammatory lesions are only found in heart tissue (7) where they are associated to parasite DNA and antigens (8,9,10), it is tempting to hypothesize that the recognition of the parasite ribosomal proteins occurs at this location. In accordance with this finding, the highest levels of antibodies against the parasite ribosomal epitope R13 were detected in patients with active myocarditis (6).

Experimental models

Interestingly, working with whole rabbit heart preparations, sera from patients with chronic Chagas' disease induced drastic modifications of the electrocardiogram (EKG) including a reduction of the node frequency and AV block (11). These effects were abolished by incubation of the human sera with synthetic peptides derived from the negatively charged C-terminal domain of the *T.cruzi* ribosomal P proteins (12,13). Remarkably, immunization with the *T.cruzi* ribosomal P2b protein (recombinant polypeptide) did not provoke heart inflammatory lesions, but induced pathological EKG with increase in the QRS complex duration, suggesting disturbances in intraventricular conduction (14).

Our results lead us to propose that in the chronic human infection the main autoreactive specificities are produced as a consequence of the cross-reacting nature of antibodies generated against parasite epitopes. We present evidence demonstrating that the so called myosin-B13 cross-reactive epitope (15), namely AALKD, does not inhibit the binding of chagasic sera to the recombinant B13 parasite protein, indicating that if anti-myosin antibodies do exist in cChHD, their specificity and cross reactive nature, if any at all, remain to be determined.

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