C0 – PARASITOLOGY YESTERDAY, TO-DAY AND TOMORROW

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The transition period between the XIXth and the XXth centuries was characterised in Parasitology by important discoveries that originated from the progress in the field of Optics and Mechanics providing new tools for the study of the microscopic level of the Biological World. We had then the description of leishmania bodies responsible for the "Orient button", the flagellate bodies in the blood of patients with malaria, followed by the description of *Plasmodium* parasites. Eggs and larvae of different helminths were identified, allowing the description of the complex life cycles of round and flat worms.

The role of mosquitoes and other Diptera and ticks, first identified as vectors of filariaisis, was extended to other pathologies in the last decade of the XIX Century. It was then possible to understand the natural history of human and animal parasitic diseases like trypanosomiasis, leishmaniaisis, malaria, schistosomiasis, filariaisis etc and to introduce rational methods for the control of parasitic diseases through the use of measures against vectors. The concomitant progress of histopathology also allowed to identify interactions of parasites with tissues and cells of the organism and to describe basic principles in the pathology of parasitic diseases. The work of Carlos Chagas, whose 90th anniversary we are now celebrating in this Congress is inscribed as among the exceptional scientific discoveries of this period in the History of Parasitology.

We are now facing an equivalent period of explosive progress in the knowledge of Parasitology. The molecular level now substitutes the microscopic level of study and we assist to rapid progress in the description of parasite genome structure. It is expected that in the next years we will have complete genome's sequences of *Plasmodium falciparum, Leishmania major, Toxoplasma gondii* and *Trypanosoma brucei*. This will be shortly followed by the description of genomes from other *Leishmania*, *Plasmodium* and *Trypanosoma* species. The present studies on "orphan" genes from Yeast will certainly facilitate the identification of new families of genes and their respective function. These progress will develop in parallel with the description of complete sequence of mouse and human genome which will allow to better understand the structure and functioning of immunological effectors and mechanisms of immune defences. Progress in structural biology and understanding of tri-dimensional structure and function of proteins and other macro molecules will permit to understand parasite- host interactions at the molecular level, which will permit the design of precise antigens and adjuvants for the construction of third and fourth generation vaccines and the production of rational drugs. This will be facilitated by the expected progress in combinatory chemistry in the synthesis of new molecules. All these progress will certainly have a paradoxical effect of inducing the disappearance of Parasitology as a defined scientific discipline. Parasitology, indeed, will become only a secondary sub-division of cell and molecular biology, which, in their turn will constitute a subdivision of macro molecular chemistry. In the expectation of this golden age, we also expect that, in our poor country, the destiny of parasitic endemic diseases, that has not changed very much from Carlos Chagas' time to now will also follows the progress for which all of us are working: the disappearance of Parasitic diseases not only as a autonomous scientific discipline but also as a dramatic social reality.

C1 – GLIDING MOTILITY AND CELL INVASION BY MALARIASPOROZOITES

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Plasmodium sporozoites and several Apicomplexa parasites suck AS Toxoplasma and Cryptosporidium glide on solid substrates. The glicind motility machinery is required for host cell invasion. This type of locomotion does not depend on glagella, cilia or pseudopodia, and its molecular basis is obscure. I will present genetic evidence that the TRAP family of surface proteins propel gliding motility. Cell invasion by Apicomplexa probably involves a capping process triggered by the attachment of the extra-cellular domains of TRAP to the surface of the target cells, and empowered by the interaction its the cytoplasmic tail with parasite motor proteins.

C2 – DIVERSITY IN THE LIPID OF THE GPI-ANCHORS IN *TRYPANOSOMA CRUZI*

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The most important glycoproteins of trypanosomatids are glycosylphosphatidylinositol (GPI)-anchored to their plasma membrane. In addition, free glycoinositolphospholipids (GIPLs) have been described, for instance the lipopeptidophosphoglycan (LPPG) which is a major component of the surface of *T. cruzi* epimastigotes.

A glycerolipid is found as constituent of GPI anchors in *T. brucei*, and *Leishmania* (review by Ferguson 1997 *Phil. Trans. R. Soc. London B* 352, 1295-1302). Thus, the first known structure, determined in the VSG of *T. brucei* is dimiristoylglycerol, unique to this parasite, and a monoacylglycerol was identified in the procyclins (PARPs). In *Leishmania* the promastigote surface protease (PSP) and the GIPLs contain an alkylacylglycerol and a lysoalkylglycerol is the lipid moiety in the LPG. In contrast, an inositolphosphoceramide (IPC) is part of the LPPG, with an anchor like structure, and of important glycoproteins present in different stages of *T. cruzi*. Crucial for the identification of ceramide was the finding that ceramide anchors could be also cleaved by bacterial PI-PLC (Lederkremer *et al*.,1990, *Eur. J. Biochem*., 192, 337-345). The lipid moieties in *T. cruzi* GPI anchors can be quite variable. However, no diacylglycerol was found in contrast with the African trypanosomes. A scheme with the major structures found was shown (Agusti *et al*., 1997, *Glycobiology*, 7, 731-735).

An alkylglycerol has been found either as a lyso species in the Tc85 glycoprotein of trypomastigotes (Couto *et al*., 1993, *Eur. J. Biochem*., 217, 597-602) or acylated as in the 1G7 anchor of metacyclic forms (Heise *et al*., 1995, *Mol. Biochem. Parasitol.*, 70, 71-84) and in the mucins of epimastigote forms (Acosta Serrano *et al*., 1995, *J. Biol. Chem.*, 270, 27244-27253). The lipid is replaced by ceramide when the parasite differentiates to metacyclic forms. Also, in the Ssp-4 glycoprotein characteristic of amastigotes, a ceramide was identified as the anchor lipid. Moreover, it was shown that extracellular differentiation of trypomastigotes to amastigotes is accompanied by an increase in the level of free ceramide in the parasite (Bertello *et al*., 1996, *Mol. Biochem. Parasitol*., 79, 143-151) suggesting cleavage of the GPI anchor by an endogenous PI-PLC activity. In the trans-sialidase of the trypomastigote stage, lyso-1-*O*-hexadecylglycerol and ceramide are present in a 1:3 ratio. In the free GIPLs of *T. cruzi* both , 1-*O*-hexadecyl-2-*O*-palmitoylglycerol and ceramide were identified in epimastigotes collected at the logarithmic phase of growth (Lederkremer *et al*., 1993, *Eur. J. Biochem*. 218, 929-936) whereas only ceramide was found in the related LPPG from epimastigotes collected at the stationary phase. Moreover, lignoceric acid is the major component in the ceramide. However, no lignoceric acid was detected when analysing the candidate precursors IPCs, in any of the stages of *T. cruzi*. All these variations suggest that a remodelling mechanism is working in *T. cruzi*.

We have now demonstrated the association of at least five phospholipase and lipase activities with the trypanosomal membranes. These enzymes may be acting in remodelling reactions leading to the anchor in the mature glycoproteins or in the LPPG. The biosynthetic pathway for the introduction of ceramide in the anchor of glycoproteins of *T. cruzi* is not known. Apparently ceramide was not described as anchor of mammalian glycoproteins. This fact, suggests that inhibition of attachment of ceramide in the anchor could be a good target for chemotherapy of Chagas disease.

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C3 – NÃO FOI ENVIADO

C4 – REGULATION OF MEDGUT TRYPSIN SYNTHESIS IN *AEDES AEGYPTI* FOLLOWING A BLOOD MEAL

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Ingestion of a blood meal induces two phases of trypsin synthesis in the midgut of *Aedes aegypti* females. The first phase, which occurs 4-6 hours following a blood meal, is characterized by the presence of small amounts of early trypsin in the midgut lumen. During this time there is limited proteolysis of the meal proteins, which is required for initiating upregulation of the transcription of several midgut genes that are essencial for complete blood meal digestion. The second phase, which occurs between 8 and 36 hours after blood feeding and involves complete digestion of the meal proteins, is characterization by the presence of large amounts of late trypsin in the midgut lumen. A speceif form of regulation of trypsin synthesis characterizes each phase: early trypsin synthesis is regulation at the translational level, while late trypsin synthesis is regulated at the transcriptional level.

Under the control of juvenile hormone, transcription of the early trypsin gene starts a few hours after adult emergence. However, despite a high steady state level, the early trypsin mRNA remains unstranslated until a blood

meal is taken. Translation is regulated at the level of aminoacylation of tRNAs in the midgut epithelium. Thus, in the unfed midgut tRNAs are largely uncharged. Within the first hour following a blood meal, tRNA aminoacylation increases due to the presence of free amino acids in blood and/or to the release of amino acids from meal proteins by exopeptidases in the midgut. In addition to early, translation of other midgut proteins within the first hours after blood feeding is essential for late trypsin transcription – early trypsin and exopeptidases work together to generate more amino acids that are used for translation of these critical proteins.

In addition to its role in amino acid production, early trypsin plays a unique and essential role in the regulation of late trypsin synthesis. While many details remain to be worked out, the following working hypothesis fits the known facts. We propose that a midgut-activating factor (MAF) is involved in regulating late trypsin gene transcription. MAF is secreted into the lumen of the midgut following feeding, binds to a midgut receptor and initiates a signal transduction pathway that involves a cAMP-dependent phosphorylation cascade. The target of this cascade is a transcription factor that is activated by phosphorylation. MAF needs to be activated by specific proteolysis by early trypsin. MAF may be stored in the midgut cells and secreted following feeding may stimulate synthesis and secretion of MAF. If there are proteins in the midgut, MAF will be protected from nonspecific proteolysis and sugnaling will occur. If here are no proteins in the midgut MAF is degraded and no signaling occurs.

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C5 – THE USE OF MATHEMATIC MODELLING OF METABOLIC PATHWAYS FOR DRUG TARGET SELECTION

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Glycolysis is the only ATP-generating process in bloodstream-form trypanosomes and therefore a promising drug target. Inhibitors which significantly decrease the glycolytic flux will kill the parasites. Both computer simulation and experimental studies of glycolysis in bloodstream-form *Trypanosoma brucei* indicated that control of the glycolytic flux is shared by several steps in the pathway. The results of these analyses provide quantitative information about the prospects of decreasing the flux by inhibition of any individual enzyme. The plasma-membrane glucose transporter appears the most promising target from this perspective, followed by aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and glycerol-3-phosphate dehydrogenase. Non-competitive or irreversible inhibitors will be most effective, but it is argued that potent competitive inhibitors can be suitable, provided that the concentration of the competing substrate cannot increase unrestrictedly. Such is the case for inhibitors that compete with coenzymes or with blood glucose. An example, using NAD analogues as specific inhibitors of the glycosomal enzyme glyceraldehyde-3-phosphate dehydrogenase and inhibiting the enzyme at sub micromolar concentrations and affecting the viability of both extracellular and intracellular trypanosomatids at low micromolar concentrations, will be presented.

C6 – MOLECULAR BASIS FOR HOST PARASITISM BY *TRICHOMONAS VAGINALIS*

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Trichomonosis is the vaginitis caused by the number one, non-viral sexually transmitted agent, *Trichomonas vaginalis*. The parasite survives in the constantly changing and adverse environment of the female urogenital tract. *T. vaginalis* cytoadhere epithelial cells preferentially. Isolation of squamous vaginal epithelial cells (VECs) and intermediate epithelial and parabasal cells demonstrated the ability of trichomonads to readily cytoadhere to VECs. The complexity of VEC cytoadherence by trichomonads is evident by the signaling for dramatic morphologic transformation that occurs within minutes after attachment. Optimal cytoadherence requires the activity of cysteine proteinases that acts uniquely on the parasite surface in an unknown fashion. Two cysteine proteinases that bind to epithelial cells and whose activity correlates with cytoadherence and cytotoxicity appear to be involved in this complex sequence of events. Four trichomonad surface proteins have been identified as mediating cytoadherence in a receptor-ligand fashion. The synthesis of the four adhesins was coordinately upregulated by binding to epithelial cells and by iron. The increased amounts of adhesins were localized to the surface adjacent to the VEC surface. Interestingly, only fresh clinical isolates, but not long-term-grown cultures, synthesized greater amounts of adhesins in response to iron. More recent work reveals that three of the four adhesins studied to date are each members of multi-gene families, and sequence analyses at both the nucleotide and amino acid levels revealed structural molecular mimicry of adhesins with known metabolic enzymes. Analysis of the receptor-binding epitope for the adhesin

AP33 identified the 24-amino acid binding domain with the ability to inhibit parasite associations with host cells. It was noteworthy that purified enzymes with identity to the adhesins were incapable of inhibiting binding of the recombinant and natural adhesins to host cell surfaces nor of preventing trichomonal cytoadherence. Finally, it is reasonable to hypothesize that trichomonosis cannot be solely explained by cytoadherence to VECs. Erosion of the vaginal epithelium as seen for colpitis macularis may allow access of parasites to the basement membrane and accompanying complex structures. Interestingly, the reports on the specific binding by *T. vaginalis* organisms to fibronectin and laminin may reflect associations with basement membrane sites. Future studies on the parasite ligands that bind these and other basement membrane components will clarify whether host parasitism involves sequential cytoadherence and association with basement membrane sites during infection. Collectively, these findings illustrate the complex and dynamic nature of infection and host parasitism by *T. vaginalis*.

C7 – RECENT IMPROVEMENTS IN CHEMICAL SYNTHESIS OG GPI MEMBRANE ANCHORS

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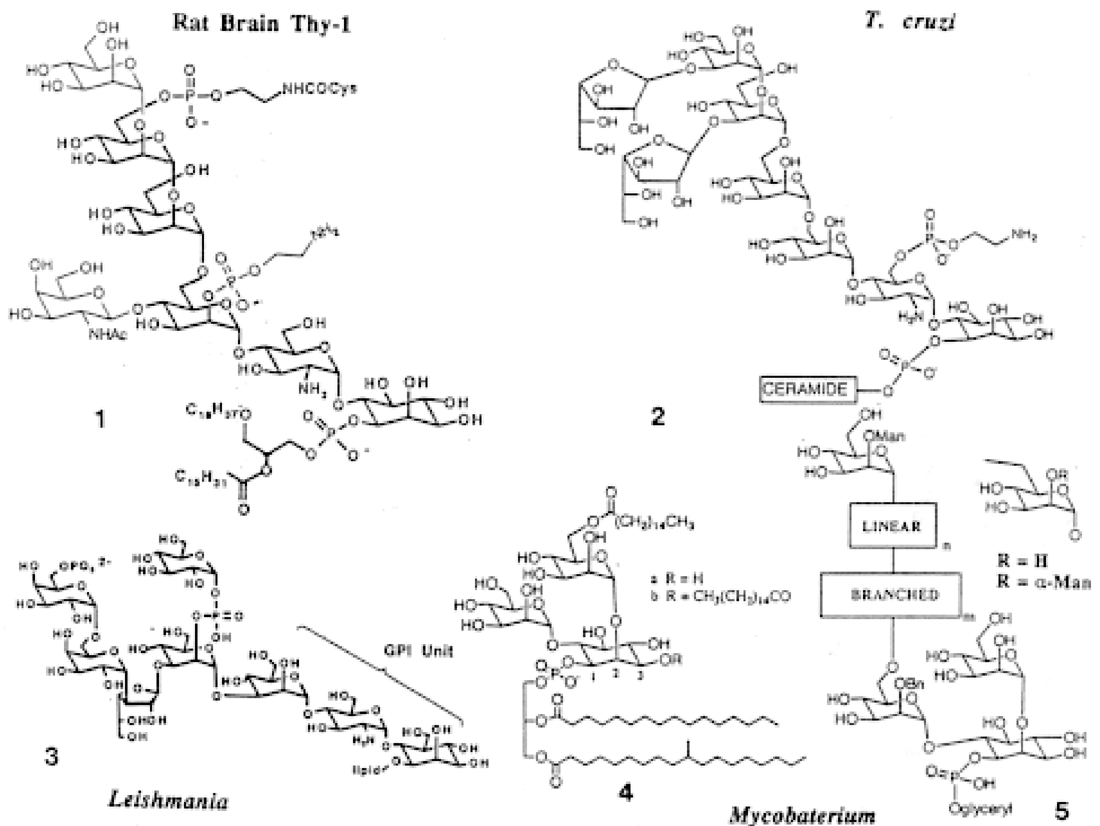
In 1995 our laboratory reported the first chemical synthesis of a GPI, 1, the rat brain Thy-1 membrane anchor (*J. Am. Chem. Soc.* 117, 10387, 1995). As the highlighted section shows, the molecule contains the classic, conserved, five-component core. Structures 2 - 5 do not contain the “classical” core, but from the standpoint of laboratory synthesis, all five structures present similar challenges. Our Institute is developing general, user-friendly strategies that should be adaptable to synthesis of both “classical” and “non-classical” GPIs.



C8 – BIOSYNTHESIS OF THE GPI-ANCHORED LIPOPHOSPHOGLYCAN OF *LEISHMANIA*: IDENTIFICATION OF THE GDP-MAN TRANSPORTER

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The glycocalyx of *Leishmania* promastigotes is composed primarily of a GPI-anchored polysaccharide called lipophosphoglycan (LPG). The *L. donovani* LPG consists of a phosphoglycan polymer of repeating Gal(?1,4)Man(?1-PO_4) units (average n=15 in procyclics; 30 in metacyclics) linked to the GPI portion [Gal-Gal-Gal_f-[Glc-PO4)]Man-Man-GlcN-]-PI. Mutants defective in the synthesis of LPG exhibit severely attenuated survival during parasite-sand fly interactions and during macrophage infections *in vitro*, demonstrating the importance of LPG as a virulence factor.

In collaboration with Dr. Stephen Beverley (Washington University), our efforts are directed toward providing a comprehensive understanding of the biochemical and genetic aspects of the biosynthesis of LPG and related glycoconjugates. LPG is an attractive parasite system for biochemical genetics as *lpg*$^-$ mutants can be rescued by functional genetic complementation, thereby permitting identification of genes involved in its assembly. Thus far, we have identified 7 such genes. One of these genes was isolated by complementation of the *L. donovani* mutant C3PO, which is unable to initiate repeating unit assembly. Functional rescue of C3PO resulted in cloning the gene *LPG2*. The *LPG2* sequence predicted a hydrophobic protein of 341 amino acids. *In vitro* studies indicated that the LPG2 protein was required for GDP-Man transport into the Golgi lumen. Heterologous expression of the *LPG2* in mammalian cells confirmed that the LPG2 protein functions autonomously to mediate GDP-Man uptake. Additional studies have indicated that the LPG2 GDP-Man transporter exists as a multimeric protein complex.

Since the GDP-mannose transporter is also shared with fungi but not mammals, inhibitors of this pathway may prove to have a broad applicability to other organisms. More generally, our studies have shown that *Leishmania* is an excellent (and for some processes, the best) system for probing basic mechanisms of glycobiology relevant to all eukaryotes.

C9 – VESICLE TARGETING WITHIN THE *T. GONDII* ENDOCYTIC PATHWAYS AND SECRETORY PATHWAYS

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Our laboratory is interested in protein targeting within the *T. gondii* endocytic and secretory pathways. Our recent results indicate that soluble proteins are routed by default to dense granules, that addition of a GPI anchor directs the proteins to a different set of vesicles delivered to the cell surface with more rapid kinetics, and that both the cytoplasmic tail and transmembrane domain of transmembrane proteins influence localization within the parasite and release into the vacuolar space. The parasite has a sophisticated system for sorting transmembrane proteins with tyrosine-based motifs in the cytoplasmic tail into at least four different compartments, including rhoptries and micronemes, via interaction with adaptor complexes in the cytosol. In conjunction with data indicating that membrane-potential-dependent styryl dyes are internalized into the parasite, this data provides evidence for endocytic membrane traffic in the organism. Using a permeabilized cell system, we have demonstrated that dense granules are released in a calcium independent fashion, via the SNAP/SNARE/Rab/NSF machinery, and that release is augmented by non-hydrolyzable GTP analogues, implicating a GTPase in the exocytosis event. The small GTP binding proteins Rab6 and Arf-1 are most likely to influence dense granule release, as determined by transient expression of dominant negative constructs of both GTPases in the parasite. In contrast, Rab 5 and Rab11 are likely to mediate endocytic membrane protein recycling to an unusual compartment anterior to the nucleus. Altogether, our results indicate that *T. gondii* uses evolutionarily conserved sorting mechanisms and protein machinery to deliver proteins to unusual organelles and unusual destinations.

C10 - VACCINE DISCOVERY AND TESTING IN A MURINE MODEL OF AMERICAN TRYPANOSOMIASIS.

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Past vaccine development efforts for *Trypanosoma cruzi* have been rather limited, in part because of the concern that autoimmunity is a primary contributor to Chagas disease. In addition, these efforts at vaccine development have met with few real successes - possibly a result of the complexity of the parasite and the immune response to it and the focus in previous vaccination efforts on the elicitation of humoral immune responses. However optimism for the development of an effective vaccine against *T. cruzi* is increased by a number of recent developments, including the presentation of strong evidence that parasite persistence and not autoimmunity is a major component of Chagas disease, the documentation of the role of $CD8^+$ T cells as primary mediators of immune control of *T. cruzi* in both mice and humans and the identification of a number of targets of parasite-induced CTL and antibody responses. Furthermore, the study of *T. cruzi* infection and the development of vaccination protocols is facilitated by the availability of excellent murine and other animal models in which both the course of infection and the development of disease can be monitored. This combination of results and attributes has led to the development of genetic immunization protocols which limit the lethality of acute infection, moderate the course of disease, and alter the course of disease when delivered therapeutically.

C11 – MOLECULAR BASIS OF TRANS-GLYCOSYLATION: STRUCTURE OF THE SIALIDASE AND TRANS-SIALIDASE FROM TRYPANOSOMES.

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Glycosidases are a large class of enzymes which hydrolyze sugar linkages but are in general unable to catalyze efficiently the inverse reaction in water. However, Trypanosoma cruzi expresses a trans-sialidase, an enzyme that is highly efficient in transferring the sialyl residue to other sugar acceptors. On the other hand, T. rangeli expresses a sialidase, 70 % identical in sequence to the T. cruzi enzyme but devoid of trans-sialylation activity. These enzymes provide a natural model system to study the mechanisms of transglycosylation, with potential applications in polysaccharide synthesis and Chagas disease treatment.

Trans-sialidase in the trypomastigote stage is encoded by about 150 genes (Cremona et al. Glycobiology, 1999; in the press) and contains two regions, the catalytic region of about 640 amino acids and a C-terminal extension made of 12-amino acid long repeats (SAPA repeats) in tandem. SAPA repeats are not required for trans-sialylation, but are necessary to maintain the enzyme active in circulation in the blood and to estimulate the production of antibodies against the catalytic domain that inhibits the trans-sialidase activity of the molecule (Buscaglia et al., J. Infect. Dis. 1998; 177,431 and Blood 1999; 93,2025). The sialidase from T. rangeli is 638 amino acid long and lacks the SAPA repeats extension (Buschiazzo et al., Glycobiology 1997; 7, 1167).

The crystal structure of the T. rangeli sialidase (TrSA) and its complex with the inhibitor 2-deoxy-2,3-dehydro-N-acetyl-neuraminic acid (DANA), was now obtained. This structure was used to model the T. cruzi trans-sialidase (TcTS) catalytic domain. The globular core of TrSA folds into two distinct domains, a canonical “beta-propeller” similar to that of viral and bacterial sialidases and a C-terminal “beta-barrel” domain. Both domains are connected by a long alfa-helical segment. Sugar residues are attached to all five predicted N-glycosylation sites, the innermost monosaccharide was modeled as N-acetylglucosamine. Comparative structural analysis of TrSA and TcTS indicates that a few amino acid substitutions around the active site confer the transglycosylation activity to the glycosidase scaffold and are responsible for the poor effect of available sialidase inhibitors on T. cruzi trans-sialidase. The relevance of these substitutions in the trans-sialidase activity were confirmed by site directed mutagenesis.

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C12 – 3-HYDROXY-3-METHYLGLUTARYL-COA REDUCTASE IN TRYPANOSOMATIDS

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Studies performed by Urbina et al. (1) have shown using a murine model of Chagas' disease that a combined treatment with mevinolin, an extremely potent competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, and azole drugs such as ketoconazole was able to essentially eliminate circulating parasites and produce complete protection against death. Likewise in *Leishmania*, the synergism of lovastatin and miconazole has been demonstrated since the combination of both drugs was more potent in terms of inhibition of promastigote proliferation, macrophage infection and amastigote numbers (2). In summary, the combined administration of ergosterol biosynthesis inhibitors that act at different points of the sterol biosynthetic pathway appears to be a promising strategy for the development of an effective treatment of leishmaniasis. The key rate-limiting enzyme of isoprenoid biosynthesis, HMG-CoA reductase, catalyzes the conversion of HMG-CoA to mevalonate, which is required for synthesis of a large family of compounds. These include cholesterol and isoprenoids such as haem A, ubiquinone, dolichol, isopentenyl tRNA, steroid hormones and other isoprenoid groups, which are covalently linked to growth-regulating proteins and oncogenic products. HMG-CoA reductase is a soluble protein in prokaryotes (3, 4, 5) while in all other eukaryotic organisms characterized, the enzyme presents a variable N-terminal membrane domain and its subcellular location appears to be preferentially the endoplasmic reticulum (6). This domain is necessary and sufficient for the regulated degradation of HMG-CoA reductase. Little is known about the nature and regulation of this enzyme in protozoa, while it has been extensively studied in mammalian cells. This regulation, which is mediated by sterols and non-sterol mevalonate-derived metabolites, is complex and occurs at many levels, including transcription, translation, protein degradation and protein phosphorylation (7). We have isolated and overexpressed the HMGCoA reductase genes from *Trypanosoma cruzi* (8), and *Leishmania major*. In both cases the enzyme lacks the membrane domain characteristic of eukaryotic cells but exhibits sequence similarity with eukaryotic reductases. Highly purified protein was achieved by ammonium sulphate precipitation followed by chromatography on hydroxyapatite. Kinetic parameters were determined for the protozoan reductase obtaining *Km* values for the overall reaction of 40.3±5.8 μM for (*R,S*)-HMG-CoA and 81.4±5.3 μM for NADPH; *Vmax* was 33.55±1.8 U mg^{-1}. Gel filtration experiments suggested an apparent molecular mass of 184 kDa with subunits of 46 kDa similar to what has been describe for certain plant reductases. In order to achieve a better understanding of the role of this enzyme in trypanosomatids, the effect of possible regulators of isoprenoid biosynthesis in cultured *Leishmania* promastigote cells was studied. Neither mevalonic acid nor serum sterols appear to modulate enzyme activity while incubation with lovastatin results in significant increases in reductase levels. This increase is reverted by the addition of the product of the reaction, mevalonic acid. Western and Northern blot analyses indicate that activation is apparently performed via post-transcriptional control.

Finally the subcellular localization of reductase was analysed. Both polyclonal and monoclonal antibodies have been generated in our laboratory against *Leishmania* and *T. cruzi* recombinant HMGCoA reductase which were used in immunolocalisation studies in wild-type parasites and mutant strains overexpressing the enzyme. Immunogold labeling of ultrathin sections of *Trypanosoma* epimastigotes and *Leishmania* promastigote forms reveals gold particles associated with the mitochondrion. These results together with digitonin precipitation experiments performed in both organisms suggest that the enzyme is predominantly located inside the mitochondrial matrix.

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C13 – PHLEBOTOMINES AND LEISHMANIA: MOLECULAR EPIDEMIOLOGICAL APPROACHES FOR UNDERSTANDING LEISHMANIASIS TRANSMISSION.

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We address herein two aspects related to phlebotomines that are of importance to leishmaniasis transmission: the rate and dispersion of ***Leishmania*** infected-sand flies, and human immune response against phlebotomine saliva.

Natural infection of *Lutzomyia spp* with *Leishmania* in endemic areas is very low. Such a low rate of detection may be due to a non-homogeneous distribution of infected vectors. We have then tested the hypothesis that there is clustering of infected vectors by combining a spatial stratification of sample harvesting of vectors with a *L. braziliensis* specific PCR and a dot blot hybridization procedure in different sectors of the Corte de Pedra area (southeast Bahia). Only *Lutzomyia whitmani* and *L. intermedia* were captured, with a great predominance of the first. A likely underestimated overall rate of 0.45% *Leishmania*-positive phlebotomines is calculated. However, approximately 80% of the positive samples were contributed by a single sector out of four sectors of the whole studied area, which provided one fourth of the total amount of analyzed samples. This resulted in a rate of 1.47% Leishmania positive phlebotomines for this sector, far above rates of other sectors. Incidence of ACL cases for this sector was about twice that for other sectors. The use of sectored analysis shows that there is a non-homogeneous distribution of *Leishmania*-infected vectors. Such a clustering may have implications in control strategies against leishmaniasis, and reinforces the necessity of understanding the ecological and geographical factors involved in leishmanial transmission.

Antibody (IgG) response to total salivary homogenate, and to a recombinant salivary protein from the sand fly *L. longipalpis* were investigated using sera from children living in an endemic area of visceral leishmaniasis (VL) in Maranhão state, Brazil. We classified children in four groups. I. Positive Serology and positive DTH. II. Positive serology and negative DTH. III. Negative serology and positive DTH. IV. Negative serology and negative DTH. When 15 sera of each of these 4 groups were tested by an ELISA assay using whole salivary gland homogenate as antigen, a statistically significant high reactivity value was found for group I. Indeed a high significant correlation was found between anti-salivary gland IgG levels and DTH response measured in mm of induration. A *L. longipalpis* salivary recombinant protein used as an antigen in an ELISA assay gave a significant, but different, result: A positive correlation was found between anti-*Leishmania* IgG and anti-recombinant protein IgG titers. Results indicate that use of sand fly salivary proteins may be of relevance to study the epidemiology of leishmaniasis. Anti-phlebtomine saliva immune response alters the course of disease in murine models, thus understanding human response to *Lutzomyia* saliva may help explain human disease.

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C14 – THE QUALITY CONTROL OF GLYCOPROTEIN FOLDING IN TRYPANOSOMATID PROTOZOA

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The lumen of the endoplasmic reticulum (ER) is the subcellular site where proteins entering the secretory pathway are glycosylated and acquire their proper tertiary structures. Proteins that fail to correctly fold are retained in the ER and eventually degraded in the proteasomes. Monoglucosylated oligosaccharides formed by glucosidase I and II (GII)-catalyzed partial deglucosylation of the oligosaccharides transferred from dolichol-P-P derivatives to nascent polypeptides ($Glc_3Man_9GlcNAc_2$) mediate glycoprotein recognition by calnexin (CNX), a membrane-bound ER-resident lectin or by calreticulin (CRT), its soluble lumenal homolog. Further deglucosylation of the oligosaccharides by GII liberates the glycoproteins from their CNX/CRT anchors. The glycans are then reglucosylated by the UDP-Glc:glycoprotein glucosyltransferase (GT), and thus recognized again by the lectins, only when linked to misfolded protein moieties as this enzyme behaves as a sensor of glycoprotein conformations. The deglucosylation-reglucosylation cycle continues until proper folding is achieved. The lectin-monoglucosylated glycoprotein interaction is one of the alternative ways by which cells retain not properly folded glycoproteins in the ER and although it decreases the folding rate, it increases folding efficiency, prevents premature glycoprotein oligomerization and degradation and suppresses formation of non-native disulfide bonds. *Trypanosoma cruzi* is the first organism in which GT activity was described. It also displays GII activity. It is now reported that this protozoan also expresses a CRT-like molecule. No CNX-encoding gene was detected. Recombinant *T. cruzi* CRT specifically recognized free monoglucosylated oligosaccharides. Addition of antiCRT serum to extracts obtained from cells pulsed with $[^{35}S]$Met only immunoprecipitated two proteins that were identified as CRT and the lysosomal proteinase cruzipain. The latter but not the former protein disappeared upon chasing cells. Contrary to what happens in mammalian cells, addition of the GII inhibitor 1-deoxynojirimycin promoted CRT-cruzipain interaction. This result is consistent with the known pathway of *N*-oligosaccharide processing occurring in *T. cruzi*. Further experiments showed that cruzipain

recognition by CRT was solely dependent on the presence of monoglucosylated glycans and not on the folding status of the protein moiety. Results obtained indicate that the quality control of glycoprotein folding appeared early in evolution. Furthermore, evidence was obtained indicating that GT glucosylated cruzipain at its last folding stages.

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C15 – 3D MODELLED OCTAMER STRUCTURE OF TRYPANOSOMA CRUZI NUCLEOSOME

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Trypanosoma cruzi presents several peculiarities in its cellular and molecular biology. One of them is the absence of chromatin condensation into chromosomes during cell division. Additionally, its chromatin is more sensitive to micrococcal nucleases and to increasing ionic strength than chromatin from higher eukaryotes.

Histones are essential proteins in the structure and function of chromatin in most eukaryotes. Accordingly, T. cruzi histone proteins and genes have seen systematically studied and characterized during the past ten years. Important differences in the amino acid sequence of the core nucleosomal histones and a histone H1 presenting only the carboxyl domain was found. With this information, and taking in consideration the crystal structure of the nucleosome core particle at 2.8 A° resolution recently described, as well as the secondary and tertiary structure conservation of histones and other related proteins, we have modelled the octamer structure of T. cruzi nucleosomes.

The tripartite assembly of the octamer (two H2A-H2B heterodimers and one H3-H4 tetramer) observed in the nucleosome of mammals is conserved in T. cruzi. However, important changes in the interactions among these three parts of the octamer, and in each of them, may explain the high sensitivity of T. cruzi chromatin to high ionic strength and to micrococcal nucleases. The observed lower stability of the octamer and the presence of a shorter histone H1 in T. cruzi nucleosomes may also explain, at least in part, the absence of chromatin condensation during mitosis in this parasite.

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C16 – MODULATION OF PHAGOSOME MATURATION DURING *LEISHMANIA* INFECTION

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Subsequent to their attachment to macrophage receptors, *Leishmania* promastigotes are internalized in a phagosome. By interacting with various endocytic organelles, the parasitophorous vacuole matures into a phagolysosome in which the promastigote transforms and multiplies as amastigote. This implies that in the mammalian host, the amastigote stage is adapted to proliferate within the acidic and hydrolase-rich environment of the phagolysosome. However, the molecular mechanisms by which promastigotes efficiently initiate infection are poorly understood. The requirement for the cell surface glycoconjugate lipophosphoglycan (LPG) repeating units in this process was evidenced by the demonstration that LPG repeating units-defective mutants are rapidly destroyed following phagocytosis. Without LPG repeating units, promastigotes are thus unable to withstand the conditions prevailing inside the maturing parasitophorous vacuole. One of the survival strategies used by *L. donovani* promastigotes during the establishment of infection in macrophages consists in inhibiting phagosome-endosome fusion. This inhibition requires the LPG repeating units, as parasites expressing truncated forms of LPG reside in phagosomes that fuse extensively with endocytic organelles. We developed a single organelle fluorescence analysis approach to study and analyze the intracellular trafficking of 'fusogenic' and 'low-fusogenic' phagosomes induced by a LPG repeating unit-defective mutant (*lpg2* KO) or by wild type *L. donovani* promastigotes, respectively. The results obtained indicate that phagosomes containing mutant parasites fuse extensively with endocytic organelles and transform into phagolysosomes by losing the early endosome markers EEA1 and transferrin receptor, and acquiring the late endocytic and lysosomal markers rab7 and LAMP1. In contrast, a majority of 'low-fusogenic' phagosomes containing wild type promastigotes do not acquire rab7 whereas they acquire LAMP1 with slower kinetics. These results suggest that *L. donovani* parasites use LPG repeating units to restrict phagosome-endosome fusion at the onset of infection in order to prevent phagosome maturation. This is likely to permit the transformation of hydrolase-sensitive promastigotes into hydrolase-resistant amastigotes within an hospitable vacuole not displaying the harsh environment of phagolysosomes.

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C17 – ULTRASTRUCTURE OF AQUATIC PROTOZOAN PARASITES: REPRODUCTION AND LIFE CYCLES

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Abstract: The large number of parasitic protozoan species itself indicates how important they are. Many species of these parasites affect aquatic animals, as molluscs, crustaceans and fishes, making them commercially less valuable and probably limit their reproduction; others lead to mass mortalities and still others may be transmitted from aquatic animal to man. The studies of the differente protozoan parasites are included in Protozoology, Protistology, or and Protoctistology. The Kingdom Protoctist, recently erected, include 7 *phyla*, most of them containing different species of parasites, some of which are pathogenic. Among these phyla, in this presentation, I shall provide an overview of some particullarities of the ultrastructure of the reproduction and life cycle of some species of the *phyla,* Apicomplexa and Myxozoa.

Phylum Apicomplexa. This *phylum* is one of the largest estimates about 5,000 described species. One of the most characteristic organelles is the apical complex found in the motile zoospores (infective stages), composed of the conoid, preconoidal ring, polar ring, subpellicular microtubules, rhoptries, and micronemes. One of the best know genus, that causes extensive, serious mortalities in their hosts, mainly bivalve molluscs, is *Perkinsus* Levine, 1978 (formerly named *Dermocystidium* sp.). This parasite appeared as a spherical walled trophozoit (meront) among gill filaments of some bivalve species. Cell multiplication occurs as result of cycles of one karyokinesis followed by cytokinesis giving rise to a morula-like cluster of some hundred of pre-zoospores. The zoosporulation occurs by differentiation, giving rise to the free biflagellated zoospores (infective cells).

Phylum Myxozoa. These parasites are characterized by spores composed of several cells transfigured into 1 to 7 spore shell values, 1 to 2 amoeboid infective germs (sporoplasm) and 2 to 7 nematocyst-like polar capsules. Spores may be of various shapes and structures. Spore dimensions with the range of 10 to 20 um long. The spore shell consists of shell valves adhering together along the lines of dehiscence or suture line, and contains the polar capsules and the sporoplasm. These parasite are coelozoic species living in body cavities of differents organs. Among the more than 1,300 recorded myxosporean species, only relatively few are know to cause letal infections. In this presentation I showed a representative myxosporean genus *Henneguya* Thélohan, 1892, that represents species infecting gills of Amazonian freshwater fishes. The spores are ellipsoidal, biconvex in sutural view and each valve continues as a caudal projection (or tail). The shell valves are smooth. Internally two polar capsules, with a coiled polar tube, are very ellongated. A binucleate sporoplasm usually with several spherical sporoplasmosomes, containing polysaccharides are present.

C18 – TRANSIENT EXPRESSION OF HETEROLOGOUS GENES IN *LUTZOMYIA LONGIPALPIS*.

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Sand flies are leishmaniasis transmitting vectors, a disease that affects more than 15 million people worldwide, with 400.000 new cases per year. They also transmit other diseases such as, bartonellosis and viral infections. Major efforts to deal with sand fly-borne diseases have focused on treatment of infected patients, vaccine development, as well as on eradicating the vector. *Leishmania* parasites developed inside sand fly midgut in a process called metacyclogenesis. The ability to express genes in sand fly would be a powerful approach to characterize midgut genes, and to reveal important vector determinants of pathogens transmission. In this context, it is important first to characterize promoters that function in sand fly cells. Since sand fly promoters have not yet been identified, the initial experiments were performed using heterologous promoters. We characterized for the first time, in New and

Old World sand fly cell lines the transient expression of the luciferase reporter gene under control of different heterologous promoters. The promoters of *D.melanogaster* heat shock protein 70 (hsp 70), human cytomegalovirus (CMV), Simian virus 40 (SV40) and *Junonia coenia* densovirus P9 (JcDNV) were recognized by the transcriptional machinery of both sand fly cell lines. Here we report the successful transfection and expression of exogenous genes in terminally differentiated sand fly organs. A lipofectin-based assay was developed to transfect *Lu. longipalpis* midguts with a DNA construct carrying the luciferase reporter gene under the control of the *Drosophila* hsp70 promoter. Luciferase expression in the midguts was detected by indirect immunofluorescence 24 hr post-transfection. The viability of the transfected midguts was the same as the non-transfected controls, as tested by ^{35}S-methionine incorporation. Epithelial cells from transfected midguts were also able to bind *Leishmania* promastigote in an *in vitro* assay. The ability to genetically transform sand flies may facilitate efforts to understand the interactions of vector-pathogens, to the extent that novel methods to control the transmission of sand fly borne diseases could be developed, or novel strategies could be generated for either eradicating the vector or for genetically converting wild insects into incompetent vectors.

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C19 – *PHYTOMONAS* SP. AS A MODEL OF TRYPANOSOMATID METABOLISM AND DRUG DESIGN

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We described previously the carbohydrate metabolism of *Phytomonas* sp. isolated from the lactiferous tubes of the latex-bearing spurge *Euphorbia characias*. It is very similar to the metabolism of bloodstream form of *Trypanosoma brucei*. In both case the mitochondrial activity has been reduced to a minimum. The citric-acid cycle is not active and a classical electron-transport chain is absent. The respiration is mediated via a mitochondrial cyanide-insensitive glycerol-3-phosphate oxidase system. Both trypanosome are unable to respire on proline, 2-oxoglutarate or succinate, and are highly dependent on carbohydrate metabolism for their energy needs. They have a very active glycolisis whose enzymes are confined to a specialised organelle, the glycosome.

We have described in *Phytomonas* sp. the presence of two malate dehydrogenase isoforms, a highly specific mitochondrial one and a glycosomal enzyme able to use oxaloacetate and other oxoacids as substrate (Uttaro and Opperdoes, 1997, *Mol. Biochem. Parasitol.* 89: 51-59). We present here evidence that the low specificity of last isoform is due to the presence of at least two highly homologous genes (gMDH2 and gHADH3) belonging to a family of 2-hydroxyacid dehydrogenases. They are 90% identical with a similar hydrophobic profile and a same net charge. However, when overexpressed in *E. coli*, gMDH2 behave as a real malate dehydrogenase but gHADH3 have activity only with aliphatic and aromatic 2-oxoacids.

In order to understand such a change of activity we made a series of recombinant between both genes. The kinetic characterisation of these overexpressed hybrid enzymes allowed us to identify aminoacids in the dehydrogenase active site involved in substrate specificity. This information will be used to design a specific inhibitor for the glycosomal malate dehydrogenase. The metabolic role of this enzyme is the reoxidation of the NADH produced during glycolysis in *Phytomonas*, *Leishmania*, *T. cruzi* and procyclic form of *T. brucei*. In the absence of this reoxidation glycolysis must come to a halt, being malate dehydrogenase a good target for the development of anti-trypanosome drugs.

C20 – SELECTING AND VALIDATING DRUG TARGETS IN THE TRYPANOSOME GPI BIOSYNTHETIC PATHWAY

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Glycosylphosphatidylinositol (GPI) membrane anchors are covalently attached to the C-terminal amino acid of many eukaryote cell-surface glycoproteins and can be considered as an alternative to a hydrophobic transmembrane polypeptide anchor. The core structure of GPI membrane anchors (ethanolamine-*P*-6Mana1-2Mana1-6Mana1-4GlcNa1-6PI) is highly conserved but the lipid component of the PI moiety, and sidechains attached to the basic core, vary in a species and tissue-specific manner (1).

The lower eukaryotes, and in particular the parasitic protozoa, express very high numbers of GPI-anchored glycoproteins and/or novel GPI-related surface glycoconjugates (1,2). The cell surface molecular architecture of these pathogens is therefore dominated by GPI-anchored structures and the GPI biosynthetic pathway represents an attractive target for the development of new therapeutic agents (2). Comparison of the African trypanosome and human GPI biosynthetic pathways has identified significant differences in the order and function of certain steps (3,4) and the application of synthetic substrate analogues has recently identified differences between the parasite and human Dol-P-Man:GlcN-PI a1-4 mannosyltransferases (4,5) and GlcNAc-PI de-N-acetylases (6).

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C21 – COINFECTION OF CELLS WITH NON VIRAL PATHOGENS, A NEGLECTED TOOL FOR THE STUDY OF MICROBIAL INTRACELLULAR INFECTION.

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Mammalian cell cultures have been coinfected with viruses or with a viral and a non-viral pathogen. However, dual infections of cells *in vitro* with non-viral, prokaryotic and/or eukaryotic organisms have only rarely been reported. We believe that coinfection experiments can be useful to: 1) investigate "direct" interactions between pathogens of different genera, species, strains, or mutants of the same organism when they coexist within the same cell. These interactions may involve competition for host cell-derived nutrients, cooperation by secretion or processing of appropriate substrates, antagonism via secreted antibiotics, or transfer of genetic elements. 2) Examine interactions mediated by pathogen effects on host cells, e.g. modulation of transduction cascades, production of protective or inhibitory chemokines or cytokines, regulation of microbicidal mechanisms or single or dual antigen expression. 3) Investigate pathogen colocalization and survival in phagosomes or cytosol, target pathogens to cell compartments they do not normally occupy, or examine compositional and functional features of chimeric phagosomes.

We will review coinfection studies that led to the construction of chimeric phagosomes [those that contain two non-viral pathogens]. Cohabitation was obtained when one of the organisms was the Rickettsia *Coxiella burnetii* (in phase II) or the flagellates *Leishmania (L.) amazonensis* or *L. (L.) mexicana*, organisms normally housed in compositionally related spacious phagolysosome-like vacuoles. On the basis of a limited array of second partners, *C. burnetii* vacuoles were more open to cohabitation than those of *L.(L.) amazonensis*. Chimeric phagosomes were built in cells coinfected with *C. burnetii* phase II and *L. amazonensis*, *T. cruzi*, or *M. avium*. (Support from FAPESP & CNPq).

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C22 – INHIBITION OF GENE EXPRESSION IN *ENTAMOEBA* BY TRANSCRIPTION OF ANTISENSE RNA

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A number of virulence factors that are responsible for the pathogenicity of *Entamoeba histolytica* have been identified and characterized in recent years. In order to investigate the role that each of these factors has, we have inhibited the expression of several genes by the transcription of their antisense RNA. Inhibition of gene expression was achieved following transfection with a hybrid plasmid construct containing the *Neo* resistance gene as well as the gene to be studied, in its reverse orientation under the regulatory elements of *EhRPg34*, one of the two *E. histolytica* genes coding for ribosomal protein L-21. The effects of inhibition of gene expression on the pathogenicity of various amoebic transfectants was investigated for the three following genes: (i) cysteine proteinase (*Eh*CP-5(; (ii) amoebapore A (AP-A), and (iii) the light subunit (35 kDa) of the Gal-specific lectin (GL). The activity of all CP's were very significantly inhibited (~90%) in the *Eh*CP-5 transfectants. Inhibition of CP activity did not affect *in vitro* cytopathic or cytolytic activity but inhibited phagocytosis of RBC's and the ability of the trophozoites to induce liver abscess formation in hamsters. Inhibition of AP synthesis (~60%) significantly affected both *in vitro* and *in vivo* pathogenicity of the transfected trophozoites and provides evidence for the importance of this component in parasite virulence. Extracts of AP^- trophozoites were also deficient in their ability to inhibit growth of *E. coli* cells and of forming pores in artificial membranes. The inhibition of the synthesis of the 35 kDa GL subunit (~60%) also had a significant effect on both the *in vitro* and *in vivo* pathogenicity of the transfected trophozoites. It is noteworthy that the synthesis of the 170 kDa heavy subunit of the Gal-specific lectin was not inhibited in the GL transfectants and their Gal-sensitive adhesion properties remained intact. The independent molecular function of the 35 kDa GL subunit in the killing of target cells is currently being investigated.

Our methods for the down regulation of gene expression by antisense RNA are helping us demonstrate the contribution of specific gene products to the pathogenesis of *E. histolytica*. Such knowledge will hopefully enable us to design better therapies against the parasite.

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C23 – PI-3 KINASE ACTIVATION, BCL-2 UPREGULATION, NF-KB TRANSLOCATION AND OTHER TRICKS PLAYED BY T. CRUZI TO INVADE CELLS.

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The Trypanosoma cruzi trans-sialidase (TS) at low doses can sensitize mice to become highly susceptible to T. cruzi invasion. In addition, transgenic Leishmania major expressing the TS of T. cruzi become extremely virulent to mice independently of enhanced Leishmania binding to macrophages and other cell types. The virulence-enhancing effect of TS in L. major was unequivocally demonstrated through the Koch's principle for assigning cause-of-infection. These observations suggest that TS is capable of sabotaging the immune system to allow T. cruzi or transgenic L. Major to thrive in mice. Indeed, we found that purified TS induces the selective release of biologically active interleukin-6 (IL-6) in naïve human intestinal microvascular endothelial cells (HIMEC), peripheral blood mononuclear cells (PBMC), and bladder carcinoma cells. in addition, TS stimulated the release of various cytokines independently of B and T cell receptor engagement, in mouse splenocytes and bone marrow cells, and in a macrophage cell line. What's more, TS served as a co-stimulatory signal for activation of naïve mouse T cells. Most interesting, HIMEC infected with a trypomastigote population expressing TS effectively released IL-6, but it did not upon infection with the counterpart trypomastigote population expressing low TS levels. The TS action was independent of its catalytic activity, as demonstrated with a genetically engineered TS mutant and an enzymatically-active polypeptide. In neuronal cells TS synergized with two IL-6 family members for protection against apoptosis induced by growth factor starvation. These novel activities of TS might, in one hand, result in the undermining of normal innate and acquired immunity against T. cruzi, and on the other hand, it might prolong parasitism by protecting cells against apoptotic death. After all, it is not in the interest of T. cruzi, nor of any pathogenic microbe, to spur the demise of their host. Molecular mechanisms for the TS actions include upregulating the oncogene Bcl-2 and activating PI-3 kinase and Akt kinase signaling pathways. But TS is not the only signal inducer of T. cruzi. We found that trypanosomes potently activates the NF-kB transcription factor to restrict T. cruzi invasion within muscle cells. However, such NF-kB-dependent muscle tropism is driven by a non-TS molecule(s).

C24 – PRE-ERYTHROCYTIC MALARIA VACCINE: MECHANISMS OF PROTECTIVE IMMUNITY AND HUMAN VACCINE TRIALS

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In order to provide a rational basis for the development of a pre-erythrocytic malaria vaccine we have aimed at: a) elucidating the mechanisms of protection, and b) identifying vaccine formulations that best elicit protection in experimental animals and humans. Based on earlier successful immunization of experimental animals with irradiated sporozoites, human volunteers were exposed to the bites of large numbers of *P. falciparum* or *P. vivax* infected irradiated mosquitoes. The result of this vaccine trial demonstrated for the first time that a pre-erythrocytic vaccine, administered to humans, can result in their complete resistance to malaria infection. However, since infected irradiated mosquitoes are unavailable for large scale vaccination, the alternative is to develop subunit vaccines. The human trials using irradiated sporozoites provided valuable information on the human immune responses to pre-erythrocytic stages and studies on mice an excellent experimental model to characterize protective immune mechanisms. The circumsporozoite protein, the first pre-erythrocytic antigen identified, is present in all malaria species, displaying a similar structure, with a central region of repeats, and two conserved regions, essential for parasite development. Most pre-erythrocytic vaccine candidates are based on the CS protein, expressed in various cell lines, microorganisms, and recently the corresponding DNA. We and others have identified CS-specific B and T cell epitopes, recognized by the rodent and human immune systems, and used them for the development of synthetic vaccines. We used synthetic peptide vaccines, multiple antigen peptides (MAPs) and polyoximes, for immunization, first in experimental animals, and recently in two human safety and immunogenicity trials. We also report here on our work on T cell mediated immunity, particularly the protection of mice immunized with viral vectors expressing CS-specific cytotoxic CD8+ T cell epitopes, and the striking booster effect of recombinant vaccinia virus. To what degree CD8+ T cells, and/or other T cells, specific for sporozoites and/or liver stage epitopes, contribute to pre-erythrocytic protective immunity in humans, remains to be determined.