S1-4 – MORBIDITY AND REGIONAL VARIATION IN CHAGAS DISEASE IN BRAZIL (*)

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The morbidity variation in Chagas disease was deeply evaluated by 32 specialists from Argentina, Bolivia, Brazil, Chile, Costa Rica, Paraguay, Peru, USA and Venezuela during a meeting on Geographic differences in Chagas disease held in Brasilia in August 1975 (Prata, 1975). This variation could be due to the virulence and pathogenicity of different T. cruzi strains and clones, to the parasite inoculum (vector capacity for infective forms) and the host-parasite interactions.

The evolutive pattern of Chagas disease is not yet completely defined and understood because its morbidity and mortality vary considerably from one area to another. Of 510 patients from different states in Brazil we observed in Rio de Janeiro (Coura et al., 1983), 39% of the cases were classified as indeterminate form, 52% were identified as cardiac form and 14% had digestive alterations (megaesophagus, megacolon or both (Fig.1). An association of cardiopathy and “megas” was observed in 10.7% of the patients and megaesophagus with megacolon in 10.9%. The highest morbidity was observed in patients from Bahia and Minas Gerais and the lowest from Rio de Janeiro and Paraíba.

Several sectional and longitudinal evaluation of the morbidity and mortality of Chagas disease have been conducted in Brazil as refered by Coura et al. (1984,a,b, 1995, 1996), Borges-Pereira (1998), Borges-Pereira et al. (1986, 1987, 1990) and Araujo (1998).

In the last 25 years we have study and followed-up the morbidity of Chagas disease in different areas in Brazil in the states of Minas Gerais, Piauí, Paraíba, Amazonas and more recently in Mato Grosso do Sul (Fig.2) which could be classified such as: a) High morbidity: Minas Gerais and Piauí, Paraiba, Amazonas and more recently in Mato Grosso do Sul (Fig.2) which could be classified such as: a) High morbidity: Minas Gerais and Piauí, b) Low morbidity: Paraíba and Mato Grosso do Sul and c) Latent chronic infection: Amazonas.

Areas of high morbidity
Follow-up studies were carried-out during 25 years in Iguatama and Pains (Area 1), Northwest of Minas Gerais, 20 years in Virgem da Lapa (Area 2) and 10 years in Berilo (Area 2A) Northeast of the state. In the first cross-sectional evaluation 264 pairs of persons from Iguatama and Pains with the same age and sex, (one with positive serology matched with one negative for Chagas disease), 274 pairs from Virgem da Lapa and 100 pairs from Berilo were studied. All pairs of persons investigated in the endemic areas were submitted to the same type of anamnesis, clinical examinations, electrocardiogram (ECG) with twelve standard leads and a chest X-ray, emphasizing the cardiac and digestive symptoms and signs. Two cross-sectional studies with the same parameters were also carried-out in the district of Oeiras (Area 3), Piauí, Northeast of Brazil, with the interval of 20 years.

The first cross-sectional study carried-out in Iguatama, Pains, Virgem da Lapa, Berilo and Oeiras, showed a serologic prevalence for Chagas infection of 17.1%, 10.4%, 12.9%, 12.7% and 12.1% respectively. The morbidity rate for Chagas cardiomiopathy (ECG gradient of seropositive/seronegative) was respectively of 23.4%, 18.4%, 19%, 22.5% and 18.2% for each aforementioned areas. Chagas disease was progressive in about 2.5 to 3% of patient/year, the mortality was more than 5 times higher in chagasic patients and the lethality was around 2% patient/year.

Areas of low morbidity
Cross-sectional studies carried-out in 8 districts of Sertão da Paraíba (Area 4) Northeast and 12 districts of Rio Verde, Mato Grosso do Sul (Area 6), Southwest of Brazil, involving, respectively, 5,137 and 14,700 persons, showed a serologic prevalence for Chagas infection of 9.5% in Paraiba and only 1.8% in Mato Grosso do Sul. The morbidity rate for Chagas cardiomiopathy (ECG gradient of seropositive/seronegative) was respectively of 23.4%, 18.4%, 19%, 22.5% and 18.2% for each aforementioned areas. Chagas disease was progressive in about 2.5 to 3% of patient/year, the mortality was more than 5 times higher in chagasic patients and the lethality was around 2% patient/year.

Latent chronic infection
Three cross-sectional studies were carried-out in the district of Barcelos (Area 5), in the micro region of Rio Negro in the northern part of the state of Amazonas, 490 Km by river from Manaus. In the first serological survey
(1991), of 710 sera tested by indirect immunofluorescence for *T. cruzi* infection, 89 (12.5%) were positive. In the second survey (1993), of 658 samples examined by the same technique 90 (13.7%) were also positive. Finally in the third survey (1997), of 886 sera analysed by the agglutination test, 117 (13.2%) were reactive, thus confirming the results obtained in the previous surveys.

The high level of seropositivity for anti-*T. cruzi* antibodies found in this study does not necessarily signify that all the cases with positive serology are actually infected with *T. cruzi*, because there is a possibility of cross-reaction with other local endemic infection such as cutaneous leishmanias and *T. rangeli*. Nonetheless, the study shows a strong epidemiological and serological correlation such as previous contact of seropositive cases with wild triatomines (20.7% of the patients recognized the triatomines which they call “piaçavas’ lice”, 67.5% of them knew the bugs from their work places being gatherers of piaçava fibers and 30% have being bitten by the bugs in their huts), isolation of *T. cruzi* by xenodiagnosis and positivity of PCR was achieved in some cases.

Electrocardiograms of 112 patients with positive serology for anti-*T. cruzi* antibodies showed 9 (8%) with alterations, 4 of them suggestive of chagasic miocardiopathy; 2 with right bundle branch block, one of these of the 3rd degree associated to left anterior hemiblock, one with A-V block of the first degree plus supraventricular extra-systoles and a disturbance of intraventricular stimulus conduction and one with bigeminal extrasystoles. However, all the patients with electrocardiographic alterations were over 60 years old which makes it difficult to exclude an association with atheroesclerosis. It is important to stress that of the 12 analysed patients older than 60, 9 (75%) had ECG alterations, which indicate a possible association of Chagas disease and heart atheroesclerosis (Coura et al. 1995).

Finally we conclude that Chagas infection in the studied area may be transmitted by wild bugs in the rural areas or in the boats during transportation of piaçava fibers. The circulating strain of *T. cruzi* is of low virulence and pathogenicity and not yet adapted to man in the dometic cycle.

References

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S3-1 – LYSOSOMES AND TRYPANOSOMES: THE REGULATION OF EXOCYTOSIS AND CELL INVASION

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_T. cruzi_ invasion of non-phagocytic mammalian cells occurs through an unusual process that involves lysosome recruitment and fusion with the plasma membrane. This process is dependent on intracellular Ca\(^{2+}\) transients triggered by the parasites in host cells (Tardieux et al. Cell 71: 1117, 1992; Caler et al. EMBO J. 17: 4986, 1998). Ca\(^{2+}\)-regulated exocytosis of lysosomes was recently recognized as an ubiquitous process in mammalian cells (Rodríguez et al. J. Cell Biol. 137: 93, 1997). Recently we showed that elevation in intracellular cAMP potentiates Ca\(^{2+}\)-dependent exocytosis of lysosomes in NRK fibroblasts. The process can be modulated by the heterotrimeric G proteins G_s and G_i, consistent with activation or inhibition of adenylyl cyclase. NRK cell stimulation with isoproterenol, a b-adrenergic agonist that activates adenylyl cyclase, enhances Ca\(^{2+}\)-dependent lysosomal exocytosis and cell invasion by _T. cruzi_, a process that involves parasite-induced [Ca\(^{2+}\)]_i_ transients and fusion of host cell lysosomes with the plasma membrane. Similarly to what is observed for _T. cruzi_ invasion, the actin cytoskeleton acts as a barrier for Ca\(^{2+}\)-induced lysosomal exocytosis. In addition, infective stages of _T. cruzi_ trigger elevation in host cell cAMP levels, while no effect is observed with non-infective forms of the parasite. These findings demonstrate that Ca\(^{2+}\) regulates lysosomal exocytosis triggered by Ca\(^{2+}\) and a parasite/host cell interaction known to involve Ca\(^{2+}\)-dependent lysosomal fusion (Rodríguez et al. J. Biol. Chem. 274: 16754, 1999). Investigating possible Ca\(^{2+}\) sensors regulating the process of Ca\(^{2+}\)-dependent lysosome exocytosis, we found that synaptotagmin VII, an ubiquitous expressed member of the synaptotagmin family of Ca\(^{2+}\) sensors regulating the process of Ca\(^{2+}\)-dependent lysosome exocytosis in NRK fibroblasts, acts as a barrier for Ca\(^{2+}\)-induced lysosomal exocytosis. In addition, infective stages of _T. cruzi_ trigger elevation in host cell cAMP levels, while no effect is observed with non-infective forms of the parasite. These findings suggest that synaptotagmin VII plays a key role in the regulation of lysosome exocytosis triggered by low micromolar Ca\(^{2+}\) in mammalian cells. Experiments are in progress to verify the effect of synaptotagmin VII domains in inhibition of lysosome exocytosis triggered by low micromolar Ca\(^{2+}\) in mammalian cells.}

S3-2 – THE USE OF A PHAGE DISPLAY RANDOM PEPTIDE LIBRARY TO SELECT PROTEIN SEQUENCES THAT INTERACT WITH MOSQUITO SALIVARY GLANDS

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The genetic manipulation of insects, aiming at reducing vectorial capacity, appeared as a new approach to the control of diseases like malaria. The idea behind this new approach is the construction of transgenic mosquitoes carrying antiparasitic chimeric constructs able to render them refractory to malaria by blocking the transmission of _Plasmodia_. One of the sites to generate a blocking agent to the parasite is the mosquito salivary glands. The parasite needs to invade this organ to be able to infect another vertebrate host during mosquito feeding. Blocking this invasion will incapacitate the mosquito to transmit the parasite.

The strategy used to block the invasion of salivary glands was the utilization of a phage display random peptide library as a source of peptides that could compete with the parasite for receptors in this organ. The library consist of M13 phages displaying 6 random peptides flanked by two cysteines (XCXXXXXXC). Phages (10\(^{13}\)) were injected into female mosquitoes hemolymph and, after a period of 30 minutes, salivary gland were dissected and washed several times in PBS buffer. Phages that remain bound to the organ were recovered by lowering the pH. These phages were count, amplified and re-injected in new females. After 4 rounds of biopanning, selected phages were isolated and analyzed.

From 40 different phage analyzed, 20% showed the same sequence (PCQRAIFQSICN), 12.5% (PCVRSVLQIIPC), 7.5% (YCQAFSYCSCA) and 5% (PCFYLGAGTPCQ). Selected phages showed a higher affinity to the salivary glands when analyzed by ELISA against total salivary glands proteins and by titering after injection into the mosquitoes.

The same experiment was repeated with an increase of the washes and just one phage was detected after 4 rounds of biopanning (MCPIWAYCRRCH). This phage shows a binding pattern to mosquito salivary glands similar to that obtained with peptides from _P. falciparum_ CS protein.

Recently a cDNA display library has been made with RNA from infected _Aedes aegypti_ midguts with _P. gallinaceum_. The library will be utilized in biopanning experiments to obtain parasite proteins that has affinity to salivary glands. This approach will give data on the process of salivary glands invasion by the parasite and a possible tool to block the transmission.

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The lipid mediators (LM) are enzymatically formed from oxidative derivates of arachidonic acid, which have a wide range of biological activities, including the role of inflammation mediators [Bozza, PT; Yu, W.; Pentrose, JF; Morgan, ES; Dvorak, AM; Weller, PE J.Exp.Med., 186: 909-20, 1997]. The arachidonic acid is abundant in cellular membranes of mammalian cells, where it is esterified into glycerophospholipids. Much stimulus activate phospholipases enzymes releasing arachidonate through the hydrolysis of the ester linkage. The arachidonate can be oxygenated by prostaglandins G/H synthase or 5-lipoxigenases to form prostaglandins (PGs), leukotrienes (LTs) or hydroxy acids [Dennis, EA BioTechnology, 5:1294-300,1987], known as eicosanoids. The eicosanoids act on different cells by binding to their respective receptors, which are thought to be G protein linked. Platelet-activating factor (PAF) is produced by a variety of cells, including neutrophils, eosinophils, monocytes/macrophages and endothelial cells [Snyder, F Am.J.Physiol., 239: C697-C708, 1990; Prescott, SM; Zimmerman, GA; McIntyre, TM J.Biol.Chem., 265: 17381-384, 1990]. The PAF biological effects are mediated by its specific receptors with the activation of several signal-transduction pathways [Wang, H; Tan, X.; Chang, H.; Gonzalez-Cruss, F; Remick, DG; Hsuheh, W. Biochem. J., 322: 603-8, 1999]. The LMs are synthetized from an outset interaction between the parasite and the host cells. It can be supposed that the different formed mediators and the host response capacity are among the factors that regulate the infection. The PGs have had a role in the exacerbation of the disease in L.m major infected BALB/c mice [Farrel, JP; Kirkpatrick, CE J.Immunol., 138: 902-7, 1987], and it has been shown that an increased production of eicosanoids occurs during the course of murine infection with L.donovani [Reiner, NE; Malemud, CJ Cell Immunol., 88: 501-10, 1984]. The infection of murine macrophages caused an increased production of PGE2 between 1-2h after infection. The addition of PGE2 to macrophage cultures increased the infection by Leishmania [Lonardoni, MVC; Barbieri, CL; Russo, M; Jancár, S Mediators Inflamm., 3: 137-41, 1994]. The endogenous leukotrienes does not seem to be involved in macrophage infection but the addition of LTs to culture inhibited the infection being mediated by nitric oxide. The PAF had a stimulatory effect upon microbicidal activity of L.(L.) amazonensis-infected macrophages. Mouse treatment with PAF-antagonists increased the paw lesion as well as the parasite load in the regional lymph nodes and in the spleen. These results suggest that PAF modulates the “in vivo” infection in mice.

**S3-4 – TOXOPLASMA GONDII AND HOST CELL INTERACTIONS: ROLE OF SOME MOLECULAR MARKERS**


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Toxoplasma gondii is one of the most widespread parasites of humans and animals. The parasite has a remarkable ability to invade a broad range of cells within its mammalian hosts by mechanisms that are poorly understood at the molecular level. This broad host cell specificity suggests that adhesion should involve the recognition of ubiquitous surface-exposed host molecules or, alternatively, the presence of various parasite attachment molecules able to recognize different host cell receptors.

The tachyzoite surface has been reported to comprise five major antigens, the most abundant of which is designated SAG1 (for surface antigen 1). At least one of the other four (SAG3) and another recently described minor antigen (SRS1 [for SAG1-related sequence 1]) have previously been shown to be structurally related to SAG1. A total of 10 members of the SAG1 gene family have now been identified, which apparently include three of the five major surface antigens previously described and one antigen expressed only in bradyzoites. The function of this family may be to provide a redundant system of receptors for interaction with host cells and/or to direct the immune responses that limit the infection. The S3-3 – THE ROLE OF LIPID MEDIATORS ON LEISHMANIA MACROPHAGE INFECTION

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The infection of murine macrophages caused an increased production of PGE2 between 1-2h after infection. The addition of PGE2 to macrophage cultures increased the infection by Leishmania [Lonardoni, MVC; Barbieri, CL; Russo, M; Jancár, S Mediators Inflamm., 3: 137-41, 1994]. The endogenous leukotrienes does not seem to be involved in macrophage infection but the addition of LTs to culture inhibited the infection being mediated by nitric oxide. The PAF had a stimulatory effect upon microbicidal activity of L.(L.) amazonensis-infected macrophages. Mouse treatment with PAF-antagonists increased the paw lesion as well as the parasite load in the regional lymph nodes and in the spleen. These results suggest that PAF modulates the “in vivo” infection in mice.
S4-1 – MUTUALLY EXCLUSIVE VAR GENE EXPRESSION IN PLASMODIUM FALCIPARUM


Cytoadhesion of P. falciparum infected erythrocytes to host receptors leads to the ‘sequestration’ of mature parasite forms in the vascular endothelium and appears to play an important role in parasite survival. It is a generally accepted dogma that sequestration initiates many of the pathogenic processes associated with severe malaria. Individual members of a large diverse gene family called var, mediate cytoadhesion and antigenic variation of infected erythrocytes. The mechanism responsible for the switching of expression between members of the var gene family has remained unresolved. The use of clonal parasite populations, selected on the basis of their cytoadherent properties, allowed us to investigate the mechanism(s) responsible for the switching of var gene expression. The early non-adhesive P. falciparum ring forms appears to transcribe virtually the entire var gene repertoire, while the adhesive trophozoite stages demonstrate mutually exclusive expression of a single var gene. Analysis of both active and inactive var gene expression sites in trophozoites demonstrates that this mechanism is controlled at the transcriptional level. Switching between different var genes, exhibiting different adhesives phenotypes, involves the in situ activation of var genes located at distinct subtelomeric or central regions, indicating the presence of multiple expression sites.

S4-2 – THE ROLE OF HOMOLOGOUS RECOMBINATION IN ANTIGENIC VARIATION IN AFRICAN TRYPANOSOMES

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Mammal-infective African trypanosomes are covered in a protective surface coat, composed of variant surface glycoprotein (VSG). The parasites undergo antigenic variation by switching the expressed VSG, thereby retaining the protection of the coat and avoiding the continuous waves of antibodies against VSGs. The trypanosome is well equipped for VSG switching, having an estimated ~1000 VSG genes, and central questions are how only one VSG is expressed at a time and how switching is achieved. VSG transcription occurs only at telomeric expression sites, of which only one is active at any time. Antigenic variation can involve transcriptional switching between these sites or a number of recombinational events, including VSG duplication, that replace the VSG in the expression site with another one. All these mechanisms have been described in laboratory-adapted, “monomorphic” trypanosomes, which are deficient in switching, by 4-5 orders of magnitude, and which preferentially undertake transcriptional switching. We have proposed that the default pathway in non-adapted, “pleomorphic” trypanosomes is gene duplication, and have now performed a large analysis of switching in such trypanosomes that reveals they do appear to be programmed for VSG duplication. A major challenge now is to understand exactly what this programme is.

The genomic environment of silent VSGs and the resemblance of VSG duplication to gene conversion suggest homologous recombination is key to the process. To begin to analyze which pathways are used, we have cloned trypanosome homologues of several genes important for recombination in other eukaryotes. RAD51, which is equivalent to bacterial RecA, is critical for DNA strand exchange at the beginning of homologous recombination. We have knocked out the trypanosomal RAD51 homologue in a marked strain of monomorphic trypanosomes in which it is possible to distinguish the overall rate of VSG switching and the particular switch mechanism used. The resultant general sensitivity to mutagens and decrease in homologous recombination suggest we have indeed cloned the orthologue of RAD51. There is a consistent decrease in the switch rate, applying to all detectable switch mechanisms, including the transcriptional switch between expression sites. The data indicate that RAD51-mediated homologous recombination accounts for most antigenic variation, and other homologous recombination pathways operate in the background. We are extending this work to pleomorphic trypanosomes and to a number of the homologous recombination pathways described in other organisms.

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When Trypanosoma brucei is ingested by the tsetse fly and transforms into a procyclic form, the VSG coat is totally replaced by a different set of abundant GPI-anchored glycoproteins known as procyclins. One type of procyclin, the EP-isofrom, has 22-30 Glu-Pro repeats at its C-terminus and is encoded by multiple genes. Because of the similarity in the predicted EP-PARP sequences, and the heterogeneity of their GPI anchors, it has been impossible to separate and characterize the EP-procyclin species by protein fraction techniques. To elucidate the role of procyclins in the parasite development in the insect vector, and to gain insight into the structure and expression of these molecules, we have analyzed and compared the entire procyclin repertoire from different procyclic cell line cultivated in vitro, using a combination of chemical treatments and mass-spectrometry techniques.

We found that one line of parasites expresses mainly the N-glycosylated products of the EP3 and EP1 genes, but only small amounts of the non-N-glycosylated products of the EP2 and GPEET genes (GPEET encodes GPEET-procyclin). These cells express two different products of the EP1 gene, EP1-1 and EP1-2, which are likely to result from the expression of two different EP1 alleles. In contrast, other trypanosomes express mostly GPEET-procyclins, with low levels of glycosylated EP-procyclin species that are polymorphic in the number of EP-repeats. Interestingly, only one gene in each procyclin locus is efficiently expressed at the protein level whereas the other is down-regulated, possibly by post-transcriptional mechanisms.

S4-4 – NUCLEAR STRUCTURE AND GENE EXPRESSION IN TRYPANOSOMA CRUZI

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The transformation of Trypanosoma cruzi from replicative (epimastigotes and amastigotes) to infective (trypomastigote) forms is accompanied by a 10 fold decrease in the transcription activity, and correlates with an intense nuclear reorganization consisting in the disappearance of the nucleolus and migration of the peripheral heterochromatin towards the nuclear interior. Here, we present experiments aiming to understand the mechanisms that control these nuclear interior. Here, we present experiments aiming to understand the mechanisms that control these nuclear modification and how these alteration are related to the differential gene expression in T. cruzi. We found that the heterochromatic regions contain the 195 bp DNA sequence repeats, know as satellite sequences, which correspond to about 10% of the entire parasite genome. These sequences form long patches of repeats (> 30 kb) in large chromosomes, and are much less transcribed in all parasite stages, as compared to other parasite genes. Differentiation into infective forms also results in an increased susceptibility of chromatin to Microccocal nuclease, but not to Dnase I, probably due to the expression of different set of histone type 1 genes. Analysis of telomeres and telomere binding proteins were also performed in different parasite stages. A telomere binding protein with 50 kDa was identified and show to be expressed mostly in replicative forms. Finally we have identified a portion of the gene corresponding to the largest subunit of RNA polymerase II of T. cruzi. This gene is similar to the one found in Trypanosoma brucei and may helpful to correlate the nuclear reorganization and the decrease in the transcriptional activity of infective forms.

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S5-1 – POWERFUL EFFECTS OF VECTOR SALIVA AND VECTOR PREEXPOSURE ON THE LONG TERM OUTCOME OF LEISHMANIA MAJOR INFECTION.

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Abstract

Despite the fact that Leishmania parasites are transmitted exclusively by the bite of an infected sand fly, there are few reports concerning the relationship of this factor on Leishmania infection. Two models were developed, one using needle injection and the second based on natural transmission by the vector.

During the natural transmission of Leishmania, sand flies inoculate low numbers of metacyclic promastigotes, along with saliva, into the skin of the mammalian host. It has previously been revealed by experimental mouse models of cutaneous Leishmianiasis that infection in a subcutaneous site is enhanced following co-inoculation of parasites with sand fly saliva. Extending these observations to a dermal model of disease, we take into account the fact that most individuals who live in endemic areas are exposed to saliva from uninfected flies before the bite that transmits infection. We developed a model of cutaneous Leishmianiasis with L. major in BALB/c and C57Bl/6 (B/6) mice that seeks to mimic these natural conditions of infection: low number of metacyclic promastigotes (1000), co-inoculation with salivary gland sonicate (SGS, 0.2 gland pair equivalents) from a natural vector. Phlebotomus papatasi, and intradermal inoculation into the ear dermis of naive mice or of mice pre-exposed to sand fly saliva.

Our studies reveal a dramatic exacerbating effect of saliva on lesion development in the dermal site, and a complete abrogation of this effect in mice pre-sensitized to sand fly saliva. In both BALB/c and B/6 mice, the dermal lesions in saliva naïve mice appeared earlier, progressed to a larger size, were more destructive, and contained greater numbers of parasites following infection in the presence of SGS. Furthermore, the dermal lesions in normally resistant B/6 mice failed to heal for up to 7 months after infection in the presence of saliva. Flow cytometric analysis permitted detection of cytokines produced by epidermal cells at the single cell level and disease exacerbation was found to be associated with the ability of saliva to elicit an early (6 hr.) increase in the frequency of cells producing Type 2 cytokines in the epidermis. The early Type 2 responses elicited by SGS in the epidermis were not observed in mice previously injected with SGS. These mice made anti-saliva antibodies that were able to neutralize both the ability of saliva to enhance infection and to elicit IL-4 and IL-5 responses in the epidermis. These results are the first to suggest that in individuals at risk of vector borne infections, the exposure history to vector saliva might influence the outcome of exposure to transmitted parasites.

The second approach was to define a reproducible murine ear model based on the transmission of Leishmania major by bite was established. The model was used to investigate the effect of host pre-sensitization to sand fly saliva via exposure to uninfected sand fly bites on the course of infection but also to analyze the initial events occurring in the site during natural transmission. This parameter is of epidemiological importance since the natural rate of infection in sand fly populations is low, and inhabitants of endemic regions are repeatedly subjected to the bite of uninfected flies before coming into contact with the parasite throughout the follow up period (P < 0.01). Moreover, 92% (11/12) of ears in naive mice developed lesions compared to 67% (8/12) in ears of sensitized mice, and the number of lesions per ear was also higher in the naïve mice. The above data clearly demonstrates an increased resistance to Leishmania as a result of host pre-exposure to uninfected sand fly bites. These results are the first to suggest that in individuals at risk of vector borne infections, the exposure history to vector saliva might influence the outcome of exposure to transmitted parasites.

S5-2 – NATURAL BARRIERS TO LEISHMANIA INFECTIONS IN THE SANDFLY VECTOR

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The completion of the life cycle of the Leishmania inside its sandfly vector is the main factor for successful of transmission to the vertebrate host. The parasite needs to deal with several challenges in the environment after being ingested by the insect. A very fine balance exists between the sandfly and the developing pathogen and there are many physical and chemical natural barriers that may interrupt the parasite’s life cycle. These barriers may lead to death of the parasite, a shortened life span or allow insufficient time for the parasite to develop and mature in order to be passed on to the vertebrate host. The ability of a Leishmania sp overcome these barriers, associated with susceptibility of certain sandfly species to infection could be determinants for vectorial capacity. The parasite needs to able to: (a) resist enzymatic activities present in the insect midgut; (b) escape from the peritrophic matrix that surrounds the bloodmeal; (c) attach to the epithelial cells; (d) develop a complete life cycle ending with differentia-
metacyclogenesis is common to all other trypanosomatids, and is essential for vector competence. In all the and demonstrated that they became progressively more virulent for vertebrates. This process known as (Am. J. Trop. Med. Hyg. 34:456, 1985) recovered promastigotes from infected midgut on days 4-7 after infection (Promastigote exist in the sandfly adapted for life in the vertebrate. However only in the 1980’s, Sacks and Perkins Since the work of Adler and Ber (Indian J. of Med. Research 29:803, 1941) it is believed that one particular form of differentiates into the infective stage, the metacyclics being the only form that is able to survive in the vertebrate host. Financial support: FIOCRUZ, WHO, CNPq and PRONEX.

tion to metacyclics, the infective form that is introduced into the vertebrate hosts by the bite of the sandfly. The first natural barrier that any microorganism including Leishmania needs to deal with is to resist or avoid the enzymatic activities in the insect midgut. The level of proteases in the gut is usually small but can rise 20-fold immediately after ingestion of bloodmeal. Adler et al (Proc. Royal Soc. London, B15:491, 1938) were the first to notice that non-susceptible species of Leishmania were killed inside the bloodmeal during the digestion in the vector species. Experiments done with different natural pairs of species of Leishmania and their sandfly vectors from New and World have demonstrated that during blood digestion the numbers of parasites inside the gut decrease from two to eight-fold, mainly due the trypsin activity (Borovsky & Schlein Med. Vet. Entomology 1:235, 1987; Pimenta et. al. Parasitology 115:359, 1997; Nieves et. al. 1999 J. Med. Entomology in press). The susceptibility of the parasite to the digestive enzymes appears to be stage specific with most mortality occurring in the intermediate forms. The completely transformed promastigotes are resistant due to the acquisition of a specific lipophosphoglycan coat on the cell surface (Schlein et al. Trans. Royal Soc. of Trop. Med. Hyg. 84: 353, 1990; Pimenta et al. 1991). The defective LPG Leishmania mutants are killed 48 h after ingestion by the vector enzymes, but in parasites with the ability to express the molecule this condition is inverted and they able to develop infection. Schlein and collaborators (Exp. Parasitology 62:376, 1986) also demonstrated that L. major is able to block a huge amount of digestive enzyme production in its natural sandfly vector, P. papatasi, this was not seen for L. donovani which is not naturally transmitted by this insect. Again the LPG molecules appears to be involved in modulating the activity of proteolytic enzymes, and therefore might delay digestion to the bloodmeal and promote parasite survival. In conclusion, several parasites are killed inside the bloodmeal due the natural physiological conditions of sandfly digestion and this factor is relating to the non-susceptible Leishmania species. The surviving parasites need to pass another barrier involving their escape from the peritrophic matrix. In blood-feeding Diptera including sandflies, the peritrophic matrix is formed immediately after the ingestion of blood. It consists of a network of chitin in a matrix composed of proteins and proteoglycans. The major roles ascribed to this structure include preventing damage or clogging of microvilli by luminal contents, compartmentalization of digestive events by acting as a permeability barrier for digestive enzyme and protection against microbes by acting as a physical barrier to their development. Loss of Leishmania infection in unnatural sandfly vectors was found to be due to the failure of the parasite to escape from the peritrophic matrix prior to bloodmeal elimination (Feng Peking Nat. Hist. Bull. 19:327, 1951; Walters et al. Am. J. Trop. Med. Hyg. 46:211, 1992), an event which is thought normally to be facilitated by the action of a parasitederived chitinase (Schlein et al. Proc. Royal Soc. of London, B245:121, 1991). In studies designed to define more conclusively the role of the peritrophic matrix in controlling the remarkable specificity of some Leishmania-sandfly interactions using sandflies fed on bloodmeals containing exogenous chitinase, a novel role for protecting Leishmania from the proteolytic activities of the blood digestion was explored by Pimenta et al. (Parasitology 115:359, 1997). The author provided direct evidence that the peritrophic matrix, while clearly acting as a potential barrier to the development of infection, also promotes early parasite survival by moderating the exposure of parasites to the hydrolytic activities of the bloodfed midgut, prior to their differentiation into protease-resistant forms. As the peritrophic matrix breaks down, the parasites need to attached to the gut epithelium to remain in the gut. In non-permissive Leishmania species the promastigote are not destroyed by the digestive enzyme or expelled with the digested bloodmeal. Within an appropriate vector, the attachment of promastigotes to the microvillar lining of the abdominal and thoracic midgut is thought to be critical. As with other trypanosomatids, this attachment to the wall of the alimentary tract allows the parasite to remain in position during the bloodmeal excretion. It has been shown that LPG is directly involved in mediating attachment of procyclic promastigotes to the midgut (Pimenta et al. Science 256:1812, 1992; Pimenta et al. PNAS 91:9115, 1994) and these experiments suggest that gut-associated lectins or lectin-like molecules, which have been described for sandflies (Wallbanks et al. Trop. Med. And Parastol. 37:409-13; 1986) serve as parasite attachment sites. After the attachment the parasite needs to multiply and differentiates into the infective stage, the metacyclics being the only form that is able to survive in the vertebrate host. Since the work of Adler and Ber (Indian J. of Med. Research 29:803, 1941) it is believed that one particular form of promastigote exist in the sandfly adapted for life in the vertebrate. However only in the 1980’s, Sacks and Perkins (Am. J. Trop. Med. Hyg. 34:456, 1985) recovered promastigotes from infected midgut on days 4-7 after infection and demonstrated that they became progressively more virulent for vertebrates. This process known as metacyclogenesis is common to all other trypanosomatids, and is essential for vector competence. In all the Leishmania species, metacyclogenesis means that the parasites become preadapted to survival within the vertebrate host while within the vector. Differentiation into metacyclic is the final challenge for the Leishmania parasite inside the sandfly vector allowing it to complete its life cycle.
S5-3 – THE STUDY OF MOSQUITO EMBRYOGENESIS IS ONE OF THE REQUIREMENTS TOWARDS THE DEVELOPMENT OF A MALARIA VECTOR CONTROL PROGRAM

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Nowadays an alternative strategy aiming at the control of malaria vectors is in development by different laboratories throughout the world. This novel strategy, based on the construction and field release of genetically modified Anopheles mosquitoes, is intended to block malaria transmission in a short period of time. To accomplish this, different research lines are being carried on, almost exclusively with Anopheles gambiae, the main african malaria vector: isolation of (a) gene(s) able to render the mosquito refractory to Plasmodium infection, characterization of gut-specific promoters, identification of mosquito transforming systems, genome mapping and isolation of mutants.

Until recently neotropical malaria vectors could not take part in this process, because free-mating colonies of these mosquitoes were not available. Since the establishment of stable (more than 60 generations) Anopheles (Nyssorhynchus) albitarsis and Anopheles (Nyssorhynchus) aquasalis free-mating colonies (J. B. P. Lima), this bottleneck is overcome. We are presently taking advantage of these colonies to unravel some relevant aspects of the subgenus Nyssorhynchus biology aiming at the establishment of this novel malaria vectors’ control program at Brazil.

One of our research lines is related to Anopheles embryogenesis. We consider that the analysis of the embryogenesis process is an additional requirement towards the establishment of this kind of vector control. It will be necessary to define time and place of the exogenous DNA injection in order to attain high transformation efficiencies. Detailed knowledge of the whole mosquito embryogenesis will also help in the characterization of mutants and transgenic lines to be generated and, consequently, in the feasibility of application of this strategy in the field. And, it is expected that data acquired with neotropical Anopheles can be applied to An. gambiae, helping transformation experiments.

The external morphological characterization of An. albitarsis embryogenesis is being carried on, by laser scanning microscopy. An adequate protocol is under development in order to perform a detailed analysis of mosquito internal structures morphogenesis. Egg permeabilization assays (based on the use of sclerotization/melanization inhibitors) are also in progress and will hopefully contribute not only to the set up of the molecular basis of embryonic development but also to the weakening of eggshell layers necessary to the exogenous DNA injection.

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S5-4 – OOGENESIS IN RHODNIUS PROLIXUS : THE ROLE OF FOLLICULAR EPITHELIUM CELLS

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The process of egg production, as in many other insects, depends on the concerted metabolic effort of different organs and tissues. In Rhodnius prolixus, the blood taken from a vertebrate, is digested and the building blocks of macromolecules, absorbed by posterior midgut and transported to several tissues where they are used to synthesize different molecules. Part of it are transformed in proteins, lipids and sugars or lipoglycoproteins, nucleic acids etc, necessary for the embryo growth. All these molecules must be transported in the hemolymph to the ovary where they are taken up by the growing oocyte by receptor mediated endocytosis. Among proteins, vitellogenin and Rhodnius Heme-binding protein (RHBP) synthesized by fat body, and Calcium binding protein (RCBP) are taken up by the oocyte. The several classes of lipids are transported by and delivered at the oocyte by a lipoprotein named lipophorin. Besides these molecules synthesized by the fat body, here we present evidences for the participation of follicle epithelial-cells as an important source of a variety of molecules such as enzymes, vitellogenin that accumulates in the oocyte and also proteins presenting multiple functions. A protein synthesized by the follicular epithelial cells, besides binding vitellogenin also protects the egg against fungi invasion. The function of this protein and the role of follicle epithelial cells on the oogenesis will be discussed.

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The resistance of mice to infection with \textit{T. cruzi} has been associated with the production of the pro-inflammatory cytokine, IL-12, which triggers the production of IFN-γ by NK and T cells. The IFN-γ produced, in turn, activates macrophages to release nitric oxide and kill the obligate intracellular amastigote forms of the parasite. TNF-α, another cytokine associated with macrophage activation provides a second signal to induce microbialicidal activity in IFN-γ activated macrophages by stimulating NO production. Since \textit{T. cruzi}-infected macrophages produce TNF-α, this cytokine appears to exert its trypanocidal activity in an autocrine fashion. The control of NOS induction is mediated by TGF-β and IL-10, both produced during the acute phase of infection.

Despite its importance as a microbicidal agent, NO is involved in the establishment and maintenance of lymphocyte unresponsiveness in mice infected with several parasites. In addition, NO induces apoptotic cell death in many different cells. In this regard, we have previously shown that splenocytes from \textit{T. cruzi}-infected mice exhibit high levels of apoptosis, which seems to be mediated by nitric oxide (NO). In the present work we investigate the role of IFN-γ in modulating NO production, apoptosis induction and host protection during \textit{T. cruzi} infection in mice. We found that the IFN-γ/− mice were highly susceptible to \textit{T. cruzi} infection, exhibited significant inhibition of NO production and apoptosis levels in splenocytes, but normal lymphoproliferative response compared to the infected WT mice. Furthermore, we found that \textit{T. cruzi} leads to an enhancement of Fas and Fas-L expression which is modulate by IFN-γ, since the IFN-γ/− infected mice showed significant lower levels of Fas and Fas-L expression. The addition of recombinant IFN-γ to splenocytes from infected IFN-γ/− mice increased apoptosis levels, Fas expression and NO production. In presence of IFN-γ and absence of NO (by addition of LNMMA), the apoptosis levels were significantly reduced, but still higher than those found in splenocytes from normal mice. In addition, Fas expression was maintained, despite the low levels of NO production, suggesting that the high levels of NO do not modulate Fas expression. Moreover, in vivo, Fas expression and NO production appear to be two distinct and independent phenomena, since the treatment of infected WT mice with the iNOS inhibitor, aminoxyguanidin, led to decreased NO and apoptosis levels but not Fas expression. Taken together, these results indicate that besides being of crucial importance in mediating resistance to \textit{T. cruzi} infection in mice, IFN-γ controls the immune response, through apoptosis modulation by mediating Fas and Fas-L expression and NO production.

Regarding to the resistance to the infection, we have investigated the role the other parasite induced-molecules, PAF and chemokines. We found that, PAF induced NO secretion by \textit{T. cruzi}-infected macrophages and the secreted NO inhibited intracellular parasite growth. The addition of a PAF antagonist, WEB 2170, inhibited both NO biosynthesis and trypanocidal activity. The iNOS/L-arginine pathway mediated trypanocidal activity, since it was inhibited by treatment with L-NMMA, an L-arginine analog. PAF-mediated NO production in infected macrophages appears to be dependent on TNF-α production since the addition of a neutralizing anti-TNF-α mAb inhibited NO synthesis. In order to test the role of PAF in mediating resistance or susceptibility to \textit{T. cruzi} infection, infected mice were treated with WEB 2170, a PAF-antagonist. These animals had increased parasitemia and earlier mortality as compared to vehicle-treated mice. Altogether our results suggest that PAF belong to a group of mediators that coordinate the mechanisms of resistance to infections with intracellular parasites.

Regarding to β-chemokines, we have previously demonstrated the expression of the MIP-1α, MIP-1β, RANTES and JE/MCP-1 mRNAs in heart of \textit{T. cruzi}-infected mice. We next investigated whether \textit{T. cruzi} trypomastigotes triggered β-chemokine mRNA expression and protein production and whether these chemokines were involved in the regulation of NO production. We found that in vivo infection with \textit{T. cruzi} led to MIP-1α, RANTES and JE/MCP1 mRNA expression by cells from peritoneal inflammatory exudate. In addition, in vitro infection with \textit{T. cruzi} resulted in β-chemokine MIP-1α, MIP-1β, RANTES and JE mRNA expression by macrophages. The expression of the β-chemokines MIP-1α, MIP-1β, RANTES and JE proteins by murine macrophages cultured with trypomastigote forms of \textit{T. cruzi} was confirmed by immunocytochemistry. Interestingly, macrophage infection with \textit{T. cruzi} also resulted in NO production, which we found to be mediated mainly by β-chemokines. Hence, treatment with anti-β-chemokine specific neutralizing antibodies partially inhibited NO release by macrophages incubated with \textit{T. cruzi} parasites. Further, the addition of exogenous β-chemokines MIP-1α, MIP-1β, RANTES and JE/MCP-1 induced an increased \textit{T. cruzi} uptake, leading to an enhanced NO production and control of parasite replication in a dose-dependent manner. L-NMMA, a specific inhibitor of the L-arginine: NO pathway, caused a decrease in NO production and parasite killing when added to cultures of macrophages stimulated with β-chemokines. Among the β-chemokines tested, JE was more potent to inhibit parasite growth, although it was much less efficient than IFN-γ. Nevertheless, JE potentiates parasite killing by macrophage incubated with low doses of IFN-γ. Together, these results suggest that in addition to their chemotactic activity, murine β-chemokines may also contribute to enhance parasite uptake and promote control of parasite replication in macrophages and may play a role in resistance to \textit{T. cruzi} infection.
We next addressed the possible role of chemokines in the pathogenesis of myocarditis that occurs in *T. cruzi*-infected mice. Then, we asked about the mediators that trigger leukocyte migration to the heart as well as the source of these possible mediators. We have previously shown mRNA expression for chemokines in the total heart cell of mice infected with *T. cruzi*, which was correlated with the type and intensity of the inflammatory infiltrate. In this work, we investigated 1) nitric oxide synthase (NOS) induction; 2) nitric oxide (NO) synthesis; 3) trypanocidal activity; and 4) chemokine and cytokine mRNA expression in isolated cardiac myocytes infected with *T. cruzi*. We found that *T. cruzi* trypomastigotes induced TNF-α, IL-1α and inducible NOS (iNOS) mRNA expression as well as NO synthesis in murine embryonic cardiac myocytes. The addition of IL-1α, IFN-γ, or TNF-α to cultured cardiac myocytes resulted in significant NO production, although the cells exhibited only low trypanocidal activity. However, simultaneous addition of IL-1α, IFN-γ and TNF-α to cultures resulted in high levels of NO, activation of iNOS and a marked trypanocidal activity. The iNOS/L-arginine pathway mediated this latter activity since it was inhibited by treatment with L-NMMA. In addition, myocardial cells infected with *T. cruzi* expressed mRNA for the chemokines KC, JE, Crg-2, RANTES and Mig. These results indicate that iNOS activation and pro-inflammatory cytokines and chemokines produced by cardiac myocytes are likely to participate control the parasite growth, cell influx and in the pathogenesis of chagasic cardiomyopathy verified *T. cruzi* infected mice.

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**S6-2 – ANALYSIS OF ANTIGENS REACTIVE T-CELLS DERIVED FROM LESIONS OF AMERICAN TEGUMENTARY LEISHMANIASIS PATIENTS**

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The higher frequency of *Leishmania* antigens reactive T-cells in lesions suggests that crucial immunological events are occurring at the site of the lesions. Immunopathological studies have contributed for characterization of the *in situ* inflammatory infiltrate, however little is known about the antigen reactivity of the T-cells present in the lesions. Our objective was to analyze the *in vitro* responsiveness of those infiltrating T-cells against different parasite antigens. Mononuclear cells obtained from lesions (LEC) or peripheral blood (PBMC) from 40 cutaneous leishmaniasis patients (CL), four mucosal patients (ML) and four patients with disseminated lesions (DissL) were phenotypically characterized for B, T, CD4+, CD8+, γδ, macrophages, IL-2 receptor (IL-2R) and IFN-γR by flow cytometry. Results showed that the percentages of T-cell subpopulations (mean±s.e) were similarly distributed in the inflammatory infiltrates from CL, ML and DissL patients. Lesions older than three months showed higher percentages of CD4+ and CD8+ T cells. The higher percentages of IL-2R (87.2±5.2%) and IFN-γR (81.3±6.6%) observed in ML as compared to CL and DissL, may be associated with the severity of mucosal lesions. Assays of lymphoproliferative responses (LPR) of LEC induced *in vitro* by *L. braziliensis* (Ag-Lb) antigens as well as by concanavalin A (Con-A), *T. gondii* (Ag-Tg) and *T. cruzi* (Ag-Tc) antigens were performed. Positive LPR to Ag-Lb (stimulation indices – SI ≥ 2.5 over the background cultures) were seen in 13 out of 24 CL patients (Δcpm x 10-3=4.9±1.8), LEC from 10 out of 16 CL patients seroreactive to *Toxoplasma* proliferate in response to Ag-Tg (Δcpm x 10-3=8.3±5). The LPR to *T. cruzi* was positive in only one out of 18 examined CL patients (SI=3.4). The supernatants of those cultures were harvest for type 1 (IFN-γ) and type 2 (IL-4, IL-5) cytokine quantification. IFN-γ production was detected in LEC cultures stimulated with Con-A, Ag-Lb and Ag-Tg. The percentages of lesions with activated LEC T-cells (IFN-γ&after *in vitro* stimulation with Con-A, Ag-Lb or Ag-Tg were respectively 100%, 85.7% and 84.6%. The LEC from seven patients with no serological evidence of past *T. gondii* infection did not proliferate or produce IFN-γ after *in vitro* stimulation with Ag-Tg. Nor IL-4 neither IL-5 were detected in the supernatants from Lb-stimulated cultures. PBMC and LEC cultures stimulated with Ag-Lb were able to produce MIP-1α e RANTES. *Leishmania*-reactive T-cells obtained after four days in cultures were also separated using a Percoll gradient for CD4+ and CD8+ phenotypic analysis. The percentages of CD4+ and CD8+ Lb-reactive T-cells were variable: three patients had CD4+ > CD8+ and three had CD8+ > CD4+. The present results show that T-cells from leishmaniasis lesions are able to proliferate and produce IFN-γ not only in response to leishmanial antigens but also after stimulation with other not related parasite antigens (Ec: Tg). Thus, T cell populations reactive to other antigens can also migrate to the site of the leishmanial lesions. Their role for healing or aggravation of the lesions is not yet well determined.

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S6-3 – CELLULAR INTERACTIONS IN THE IMMUNOPATHOLOGY OF MALARIA

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The main pathological manifestations of malaria result from the ability of plasmodia to grow within erythrocytes, and particularly, from the interplay of the parasite and its antigens with the immune system. The consequences of these interactions are anemia, pulmonary edema, renal dysfunction and neurological abnormalities, which cause over one million deaths a year. Malaria-associated anemia is due to multiple and complex factors, including the mechanical destruction of erythrocytes by the growing parasite, alterations of erythropoiesis, spleen sequestration of erythrocytes, and blood loss as a consequence of the coagulopathy. The expression of parasite antigens or neo-antigens in the membrane of erythrocytes elicits different mechanisms of anemia related to the action of antibodies (including auto-antibodies), complement and phagocytosis by macrophages. The peculiar open circulation of blood in the spleen adds an extra hazard to erythrocyte survival in malaria. To return to the venous sinuses erythrocytes have to squeeze themselves through the narrow interendothelial gaps. Parasitized erythrocytes make a slower cross, which causes splenic stasis, and may be deparasitized during this event. Released parasites are phagocytosed by red-pulp macrophages, while deparasitized erythrocytes (espherocytes or ropalocytes) return to the circulation as cells with a shortened mean life. Two kinds of cellular interactions play a crucial role in the immunopathology of brain, lung and kidney involvement in malaria: parasitized erythrocytes with endothelial cells, and macrophages with these cells. Binding of parasitized erythrocytes (e.g. by Plasmodium falciparum) to endothelial cells (and to unparasitized erythrocytes) involves different adhesion molecules and receptors, and represents a critical step for the life cycle of the parasite. It is possible that the particular microenvironment generated by this sequestration facilitates the local activation of monocytes and lymphocytes, and the subsequent release of inflammatory cytokines (e.g., TNF-α, IL-6), which may activate endothelial cells, and eventually damage them. The ability of injected recombinant TNF-α to cause several pathological features of severe malaria, together with the finding that the plasma levels of this cytokine are usually elevated in infected individuals were considered as possible evidence of the responsibility of this cytokine in the pathogenesis of malaria. However, the attempts to interfere with the pathogenesis of malaria by inhibiting TNF-α synthesis with drugs (e.g. pentoxifylline), or its action with monoclonal antibodies have achieved frustrating results. Since it is recognized that hyperactivation of the immune system is associated with the pathological manifestations of severe malaria, it can be anticipated that a better understanding of the interactions of macrophages and lymphocytes, and of these cells with endothelial cells and parasitized erythrocytes, will open new avenues for the adoption of novel methods for reducing the morbimortality of malaria.

S6-4 – ROLE OF NF-KB IN THE REGULATION OF INNATE AND ADAPTIVE IMMUNITY TO TOXOPLASMA GONDII

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T. gondii is an opportunistic pathogen in patients with AIDS as well as patients with other defects in T cell mediated immunity and remains an important cause of congenital disease. The NF-kB transcription factors are an ancient family associated with the regulation of innate immunity to infection. These transcription factors can regulate the production of factors (IL-1, IL-2, TNF-α, IL-12, IFN-γ, iNOS) and the responses of immune cells to stimuli (IL-1, TNF-α, IL-12, CD28) involved in resistance to T. gondii. In addition, the activation of NF-kB in response to cytokines enhances replication of HIV-1 and it has been proposed that during the immune response to opportunistic infections there is activation of NF-kB and increased replication of HIV which hastens the course of disease. However, there are few studies which have examined the role of NF-kB in resistance to the opportunistic infection taht affect patients with AIDS. Our in vitro and in vivo studies have shown that there are high levels of NF-kB activation following infection with T. gondii. The functional significance of this observation was shown by our studies with mice deficient in different NF-kB family members. We found that the NF-kB family member RelB is required for the ability of T cells to produce IFN-γ and activation of NK cells after infection with T. gondii whereas NF-kB2 appears to be involved in the maintenance of the immune response to this parasite. We hypothesize that the activation of NF-kB represent the very earliest elements of the innate response to T. gondii. These studies address fundamental questions about the role of NF-KB in the regulation of the innate immune response and may help to understand the molecular and cellular basis of these factors in resistance to the opportunistic infection that affect patients with AIDS.
S7-1 – FUNCTIONAL ANALYSIS OF THE GPI:PROTEIN TRANSAMIDASE OF LEISHMANIA MEXICANA

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Glycosylphosphatidylinositol (GPI) anchors are used as a means of tethering surface proteins to the plasma membrane in all eukaryotes, but are particularly abundant among parasitic protozoa such as *Leishmania*. For example, the major surface protein of *Leishmania* is a 63 kDa metalloproteinase (gp63) that is attached to the surface membrane with this type of lipid anchor. The anchors are added to precursor proteins within the endoplasmic reticulum by a process that involves a proteolytic cleavage followed by a transamidation event. This is carried out by a multi-component complex called the GPI:protein transamidase. We have cloned from *Leishmania mexicana* a single copy gene, GPI8, which encodes the catalytic subunit of the transamidase complex. We have cloned from *Leishmania mexicana* a single copy gene, GPI8, which encodes the catalytic subunit of the transamidase complex. GPI8 is a member of the C13 family of cysteine peptidases.

We created GPI8 null mutants (DGPI8) through targeted gene replacement. Surface-located gp63 is absent from DGPI8 mutants; episomal-expression of GPI8 restores gp63 to the cell surface. DGPI8 mutants are viable as promastigotes in culture and are capable of differentiating into amastigote-like forms \textit{in vitro}. In addition DGPI8 mutants can invade and proliferate in macrophages \textit{in vitro}, which shows that GPI-anchored surface proteins are not essential for entry into and survival of *L. mexicana* within host cells.

DGPI8 mutants do not express detectable levels of any GPI-anchored proteins and accumulate a putative protein-anchor precursor and an inositol-acylated form of this precursor. Pulse chase labelling suggests that this acylated compound is not an intermediate in protein-anchor biosynthesis but rather a metabolic end-product. The cellular levels of two classes of non-protein-linked GPIs, a lipophosphoglycan (LPG) and a family of free GPI glycolipids (GIPLs), are not altered in the DGPI8 mutant.

S7-2 – THE MYSTERIOUS GPI-PLC WHICH RELEASES THE VSG OF AFRICAN TRYPANOSOMES

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African trypanosomes are coated by 10 million copies of a single variant specific glycoprotein (VSG) which are held in the plasma membrane by glycosylphosphatidylinositol (GPI) anchors. A GPI-specific phospholipase C (GPI-PLC) triggers fast VSG release upon cell lysis but in vivo it is safely controlled and topologically concealed from its substrate by being intracellular (Bülow et al., 1989, J. Cell Sci. 93, 233-240). One enigmatic aspect of GPI-PLC action therefore consists of how it could gain access to the VSG in the exoplasmic leaflet of the membrane.

The data to be presented disclosed an unexpected possible solution for this puzzle: upon cell rupture the VSG invades the cytoplasmic face of the plasma membrane which thus, becomes double coated. This unusual VSG arrangement was stable in ruptured plasma membrane from GPI-PLC null mutant trypanosomes but was transiently seen preceding VSG release by GPI-PLC in wild type (WT) trypanosomes. The formation of double coat membrane (DCM) was independent of the presence or activation of GPI-PLC, occurred both at 4\textdegree C and 30\textdegree C and was unaffected by the classical inhibitor of VSG release, p-chloromercuribenzenesulfonic acid (PCM). DCMs conserved the same membrane coat thickness and association with subpellicular microtubules as in intact cells, but were prone to form vesicles following gradual detachment of the latter.

Our data also demonstrated that: (i) GPI-PLC expressed by one trypanosome only targetted its own plasma membrane, being unable to release VSG of another parasite; (ii) DCMs concomitantly formed from trypanosomes expressing different VSGs did not intermix, an indication that DCM might be refractory to membrane fusion; (iii) in vivo both membrane form and GPI-PLC-cleaved molecules were detected in the lumen of the flagellar pocket.

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However, ether-lipid analogues were shown to be extremely toxic against biosynthetic pathways as suitable targets for chemotherapeutic intervention in human-pathogenic species. 

A recent report (Eintracht, J., Maathai, R., Mellors, A. and Ruben, L., 1998, Biochem. J. 336, 659-666) proposed, based on the stimulation of calcium entry by both the phospholipase A2 activator melittin and unsaturated fatty acids, and its inhibition by phospholipase A2 inhibitors, that calcium entry in *Trypanosoma brucei* bloodstream trypomastigotes is regulated via a signaling pathway involving phospholipase A2-mediated generation of arachidonic acid and stimulation of a plasma membrane-located calcium channel. Here we show that Ca\(^{2+}\) influx in *Trypanosoma brucei* procyclic trypomastigotes, *Leishmania donovani* promastigotes and *Trypanosoma cruzi* amastigotes is also stimulated in a dose-dependent manner (50-400 nM) by the amphiphilic peptide melittin. This effect is also blocked by the phospholipase A2 inhibitor 3-(4-octadecyl)-benzoylacrylic acid (OBAA). The unsaturated fatty acids arachidonic, linolenic, and linoleic acid, in the range of 10-75 mM, also induced Ca\(^{2+}\) entry by a mechanism sensitive to LaCl\(_3\). However, stimulation of Ca\(^{2+}\) influx by myristic acid as well as Ca\(^{2+}\) and H\(^+\) fluxes across the membranes of mitochondria and acidocalcisomes by these unsaturated and saturated fatty acids, in the same concentration range that stimulates Ca\(^{2+}\) entry into the cells, and the protective effect of LaCl\(_3\) on the decrease of the mitochondrial membrane potential caused by arachidonic acid, raise some doubts about the mechanism of such stimulation of Ca\(^{2+}\) transport. Although the present data do not rule out the possibility of a signaling pathway involving Ca\(^{2+}\) entry via phospholipase A2-mediated generation of free fatty acids, other possibilities for the stimulation of calcium transport through the plasma membrane and intracellular compartments such as transmembrane flip-flop of the fatty acid-cation complex or cation leakage due to fatty acid-induced bilayer packing defects might also be considered.

One of the common features of all trypanosomatids is the presence of high amounts of glycoconjugates linked to the surface via glycosylphosphatidylinositol anchors. The lipid moieties of these anchors may include lyso-acyl- and di-acyl-glycerolipids, ether lipids in the form of lyso-alkyl- and/or alkyl-acyl-glycerolipids, sphingolipids or both ether lipids and sphingolipids. Despite the extensive compositional analyses done so far with several species and the studies of fatty acid remodelling and lipid exchange in *Trypanosoma brucei*, little is known about the mechanisms of ether-lipid and sphingolipid biosynthesis. In a first round, we will consider the potential use of these biosynthetic pathways as suitable targets for chemotherapeutic intervention in human-pathogenic species.

The three initial steps of ether-lipid biosynthesis are associated with glycosomes of *T. brucei* and *Leishmania* species and, as described for mammalian cells, the synthesis follows the dihydroxyacetonephosphate pathway. So far, a direct connection of ether-lipid synthesis and GPI anchor formation has not been demonstrated in *Leishmania*. However, ether-lipid analogues were shown to be extremely toxic against *Leishmania* and have been selected by the WHO for clinical trials against leishmaniasis. Toxicity, but at a lower level, has also been observed against *Trypanosoma* spp. The exact reason for this toxicity in not known but it has been suggested that the mode of action of these analogues would be the result of a perturbation of GPI-anchor biosynthesis, phospholipid biosynthesis and/or disturbance of signal transduction by inhibition of protein kinase C. Because of these observations, the glycosomal ether-lipid biosynthetic pathway may serve as a suitable drug target.

The initial steps of sphingolipid biosynthesis in fungi and animal cells are conserved. However, the description of selective inhibitors suggested functional differences between the enzymes present in the two groups. Therefore, those enzymes became excellent targets for the action of new and potent anti-fungal chemotherapeutic agents. In addition, fungi do not form sphingomyelin like mammalian cells. Instead, fungi carry out the transfer of a phosphatidylinositol moiety to the hydroxyl group of carbon 1 of ceramide for the production of inositolphosphoceramide. Because of the similarity in the production of several components containing inositolphosphoceramides and of the chemical characteristics described to date, the sphingolipid biosynthetic pathway may also be explored as a putative chemotherapeutic target against *T. cruzi*.
**S8-1 – THE INTRACELLULAR TRANSPORT OF PROTEINS IN PLASMODIUM FALCIPARUM**

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During the intraerythrocytic cycle, *Plasmodium falciparum* synthesis several proteins that are exported beyond its membrane. These secreted antigens represent an important virulent factor by modifying the morphological and antigenic properties of the red blood cell. The mechanisms and pathways involved in proteins trafficking are poorly understood. In order to better understand the mechanism implicated in protein secretion by the parasite, we analysed the transport of the histidine-rich protein I (PfHRPI), the exported protein 1 (exp-1) and the 41-2 antigen. The exp-1 and 41-2 antigens are associated to membranous structures in the red blood cell cytoplasm, whereas PfHRPI is located at the erythrocyte’s membrane. Synchronised parasite cultures were treated with Brefeldin A (BFA), processed for immunofluorescence and visualised on a confocal laser scanning microscope. Our results show that the transport of 41-2 and exp-1 is blocked by BFA while that of PfHRPI is insensitive to the drug. This data suggests that PfHRPI is secreted through an alternate pathway, different from the ER-Golgi apparatus. We further analysed the secretion of these antigens by *in vitro* translation/translocation studies. The genes coding for the three antigens were cloned into the pBluescript vector, transcribed and translated with a rabbit reticulocyte lysate in the presence or absence of canine pancreatic microsomal membranes. As expected, exp-1 is translocated and PfHRPI is not translocated into the microsomes. The 41-2 antigen does not cross the microsomal membranes in contradiction with the results obtained *in vitro* with the BFA-treated parasites. The 41-2 translocation might occur post-translationally in the heterologous system. Our results suggest that *Plasmodium* possesses two secretion pathways: the classical pathway represented by the ER-Golgi apparatus and an alternate, BFA-insensitive, not characterised pathway. This later may represent and important target for the development of new drugs against the parasite.

**S8-2 – THE ENDOCYTIC PATHWAY IN Trypanosoma cruzi EPIMASTIGOTES**

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It has been known for many years that the trypanosomatids require exogenous essential growth factors such as heme and cholesterol in order to divide. Additional data showed that they are able to take in exogenous macromolecules such as ferritin, peroxidase, albumin, etc and that two structural components are involved in starting this process: the flagellar pocket and the cytostome. Although the flagellar pocket plays a fundamental role in the endocytic process occurring in several trypanosomatids, the cytostome is the main structure involved in such process in epimastigote forms of *T. cruzi*. One-minute endocytosis of gold labeled tracers revealed very few cells with a small number of particles inside the flagellar pocket, whereas more than 98% of the cells analized presented the markers entering the cytostome. Freeze-fracture studies showed that it appears as a specialized, smooth region of the plasma membrane very poor in intramembranous particles, but limited by a row of closely associated particles. Using the fracture-flip technique it has been shown that the actual surface of the cytostome is rugous containing a material rich in carbohydrates which can be revealed by ruthenium red and several lectins. After two, five and fifteen-minute endocytosis, gold particles were seen inside vesicles and tubules, prior to fusing with reservosomes. Three-dimensional reconstruction of these tubules and vesicles showed they are interconnected, forming an intrincated and branched network, distributed from the perinuclear region to the posterior end of the cell. Whole unfixed parasites that had uptaken gold-protein conjugates for fifteen minutes were washed and dried onto electron microscope grids. Observation with energy loss filtering TEM revealed long gold-filled tubules at the cell posterior end. The acidic nature of the early endosomal network was verified using acridine orange. Based on pH and protein uptake kinetics we propose that the vesicle-tubular network is the early endosome of *Trypanosoma cruzi* epimastigotes.

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S8-3 – ACIDOCALCISOME: A NOVEL CALCIUM STORAGE COMPARTMENT IN PROTOZOAN PARASITES

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In all eukaryotic cells, acidification is driven by ATPases of the vacuolar type. Additionally, some cell types have H⁺-pumps which are driven by pyrophosphate. Vacular H⁺-pyrophosphatases (V-H⁺-PPases) had, until recently, been found mainly in vacuoles of plants, ranging from the unicellular alga Acetabularia to higher plants (1). The known range of organisms possessing V-H⁺-PPases was recently greatly expanded by our discovery of this activity in Trypanosoma cruzi (2). Our results showed that much of the activity was associated with a vesicle rich in calcium, phosphorus, and magnesium, which we had previously identified as the acidocalcisome (3). This organelle was first described in T. brucei (4). We initially defined the acidocalcisome in permeabilized T. brucei functionally, as an organelle that was acidic and that imported Ca²⁺ by the action of a vanadate-sensitive Ca²⁺-ATPase. Subsequently, acidocalciosomes were detected in other trypanosomatids, i.e., T. cruzi (5), and Leishmania amazonenssis (6), and in the apicomplexan parasite Toxoplasma gondii (7). In recent work, we found that both bloodstream and procyclic trypomastigotes of T. brucei (8), as well as L. donovani promastigotes (8), T. gondii tachyzoites (9) and Plasmodium berghei trophozoites (10) possess a V-H⁺-PPase with features in common with the acidocalcisome. Chemical, phosphorus, and magnesium, which we had previously identified as the acidocalcisome (3). This organelle was first described in T. brucei (4). We initially defined the acidocalcisome in permeabilized T. brucei functionally, as an organelle that was acidic and that imported Ca²⁺ by the action of a vanadate-sensitive Ca²⁺-ATPase. Subsequently, acidocalciosomes were detected in other trypanosomatids, i.e., T. cruzi (5), and Leishmania amazonenssis (6), and in the apicomplexan parasite Toxoplasma gondii (7). In recent work, we found that both bloodstream and procyclic trypomastigotes of T. brucei (8), as well as L. donovani promastigotes (8), T. gondii tachyzoites (9) and Plasmodium berghei trophozoites (10) possess a V-H⁺-PPase with features in common with the acidocalcisome. Together, these data suggest co-localization of H⁺-ATPase and H⁺-PPase activities in T. brucei and provide evidence that the isolated acidocalcisome is the same organelle as that identified initially on a functional basis.


S8-4 – CA²⁺ SIGNALING IN PARAMECIUM DURING SYNCHRONOUS TRICHOCYST EXOCYTOSIS

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In the ciliated protozoan, Paramecium tetraurelia, polyamines like aminoethylxetran (AED) can stimulate synchronous exocytosis of dense core secretory vesicles (trichocysts) within 80 ms. This depends on an increase of cortical free Ca²⁺ concentration, [Ca²⁺], which is the result of a massive increase of total Ca concentration [Ca]. Both these components have been analyzed, (i) [Ca²⁺] by injected fluorochromes, eventually using fast confocal laser scanning microscopy (CLSM) from 33 ms (image build-up time) on, and (ii) by quenched-flow combined with freeze-substitution and energy-dispersive x-ray microanalysis (EDX) attached to a scanning transmission EM (STEM), for analysis from 30 ms (dead time) on. In both cases, analyses were run with or without extracellular Ca²⁺, Ca²⁺-stores are depleted by ~50 % already within 80 ms AED stimulation. With Ca²⁺ added, store depletion is immediately superimposed by Ca²⁺ (or Sr²⁺) influx into the stores, also within 80 ms. This may optimize signal response by site-directed Ca²⁺ flux during stimulation.

To determine [Ca] transients, samples were mixed with AED +/- Ca²⁺ (normally 500 µM, eventually substituted for by Sr²⁺) for different sub-second time intervals and rapidly cryofixed by spraying into liquid propane, followed by freeze-substitution with fluoride (to retain Ca or Sr). 500 nm thick sections were analyzed by EDX. Element-specific Caₐ, Srₐ signals were quantified after calibration. Under resting conditions we find Ca signals selectively in alveolar sacs. Without Ca²⁺, Ca²⁺-stores are depleted by ~50 % already within 80 ms AED stimulation. With Ca²⁺ added, store depletion is immediately superimposed by Ca²⁺ (or Sr²⁺) influx into the stores, also within 80 ms. This may optimize signal response by site-directed Ca²⁺ flux during stimulation.

described in CD4+ T cells during the acute phase of experimental Chagas’ disease (3, 4). The onset of AICD in parasite and play a decisive role in the host response to infection (2). Activation induced cell death (AICD) has been of people (1). In experimental Chagas’ disease, cytokines regulate the ability of infected macrophages to kill the addition, both intact anti-α and anti-β mAbs were able to induce T. cruzi replication in infected macrophages, in the absence of apoptotic cells.

PGE2 and TGF-β have been reported to inhibit pro-inflammatory cytokine production by human macrophages treated with apoptotic cells (7). We found that prostaglandin antagonists blocked the pro-T. cruzi activity of apoptotic cells in vitro and almost completely eliminated parasitemia after in vivo injection in infected mice. Apoptotic, but not necrotic T cells, induced intense PGE-2 and TGF-β production by both uninfected and infected macrophages. High levels of PGE-2 and TGF-β were also detected following engagement of VnR by anti-α, mAb. Neutralization of TGF-β blocked T. cruzi growth in a dose-dependent manner. It has been demonstrated that TGF-β deviates arginine metabolism from NO to both urea and ornithine production, by inducing arginase activity (8). Ornithine is a necessary precursor for putrescine synthesis by ornithine decarboxylase (ODC). ODC is expressed by the host, but not by T. cruzi. We found that apoptotic T cells induced high levels of ODC activity and putrescine production in macrophage cultures. To investigate the involvement of host-derived putrescine in parasite replication, a competitive ODC inhibitor, a-methylornithine (MO), was added to cultures, and suppressed the apoptotic cell effects on T. cruzi growth.

These results suggest that ongoing T cell apoptosis and phagocytosis of apoptotic bodies by macrophages can be one of the mechanisms involved in parasite persistence in Chagas’ disease. In addition, widely employed Prostaglandin antagonists could be helpful to control T. cruzi burden and to reduce parasite-dependent consequences on chronic Chagas’ disease.

Summary. Balance calculations from results obtained by the widely different approaches indicate that the cell operates with an excess by orders of magnitude of Ca. The anatomical arrangement of subcellular and molecular components guarantees maximal effect on the respective targets.

References

Trypanosoma cruzi is the causative agent of Chagas’ disease, a debilitating heart disease, which affects millions of people (1). In experimental Chagas’ disease, cytokines regulate the ability of infected macrophages to kill the parasite and play a decisive role in the host response to infection (2). Activation induced cell death (AICD) has been described in CD4+ T cells during the acute phase of experimental Chagas’ disease (3, 4). The onset of AICD in CD4+ T cells increased T. cruzi growth in cocultured macrophages (5), and blockade of Fas-FasL interaction inhibited both cell death and parasite replication (5). These results prompted us to investigate the direct effects of apoptotic T cells on macrophage capability of dealing with T. cruzi.

Apoptotic, but not necrotic cells exacerbated T. cruzi growth in macrophages either infected in vitro, or derived from infected mice. In agreement with in vitro results, in vivo injection of apoptotic, but not necrotic T cells in T. cruzi infected mice increased parasitemia. In order to investigate if apoptotic bodies interfere within macrophage trypanocidal activity, in vitro experiments were conducted with macrophages activated by INF-γ and LPS. Apoptotic T cells increased T. cruzi replication even in macrophages activated by those potent NO synthase inducers. Moreover, NO production induced by INF-γ/LPS was significantly reduced by apoptotic T cells.

Experiments with RGDS and RGDE peptides identified an important role for an integrin in the uptake of apoptotic T cells by infected macrophages. Moreover, RGDS, but not RGES, inhibited parasite growth induced by apoptotic T cells. We then, tested the role played by the Vitronectin Receptor (VnR or αvβ3 integrin). Fab fragments from an anti-α and anti-β mAbs were able to inhibit both apoptotic cell binding and apoptotic cell-driven T. cruzi growth in macrophages. In addition, both intact anti-α and anti-β mAbs were able to induce T. cruzi replication in infected macrophages, in the absence of apoptotic cells.

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The surface of the protozoan parasite Trypanosoma cruzi, the causative agent of Chagas’ disease (American Trypanosomiasis) in man, displays a unique biological system comprised both by an enzyme known as Trans-sialidase (TS) and by sialic acid acceptor molecules known as sialoglycoproteins or mucins like molecules (1-3). This functional complex is thought to play an important role in host-parasite interaction (4). Recent studies demonstrated that, besides a role in mammalian cell invasion, the soluble form of TS also functions as a virulence determinant molecule (5), and therefore, could have relevant biological effects on the host immune system. The studies by Chuenkova and Pereira (5) showed that in vivo injection of minute amounts of purified TS increased subsequent parasitemia and mortality in T. cruzi-infected mice. The mechanisms responsible for these effects were not determined but, since TS injection into immunodeficient SCID mice did not affect parasitemia or mortality, it was suggested that TS acts on host lymphocytes of the acquired immune system (5). T lymphocytes bearing conventional αβ TCRs are required for control of parasitemia and mortality in murine infection by T. cruzi (6). In order to better understand molecular mechanisms of Chagas’ disease, it is important to investigate the effects of TS on host T-lymphocyte functioning, and to correlate these effects with immunopathological changes induced by the parasite.

In the present study, we show that native and recombinant TS exert multiple stimulatory effects on host CD4+ T lymphocytes. TS activates CD4+ T cells in vivo and in vitro. An in vivo injection of TS increased CD44 expression on CD4+ T cells from draining lymph node. In vitro studies showed that, in the presence of a costimulus, TS induced mitogenic responses and synergized with TCR stimulation for activating CD4+ T cells from normal BALB/c mice. By immunoblotting, TS induced ERK-1/ERK-2 nuclear translocation, and synergized with TCR signals to enhance MAP kinase translocation. TS induced maximal TNF-α secretion in the absence of any TCR stimulation and increased IL-2 secretion and by TCR-activated CD4+ T cells. Exposure of CD4+ T cells from T. cruzi-infected mice to TS also induced co-mitogenic response, and completely blocked activation-induced cell death (AICD) triggered by TCR ligation. Following infection with T. cruzi, surface expression of CD43 was upregulated in CD8+ T cells and in a subset of CD4+ T cells. Pre-treatment of T cells with TS prevented the binding of anti-CD43 mAb, but not the binding of mAbs to CD4, CD8 or to CD45, another sialylated protein. CD43 ligation blocked TCR- 

S9-2 – TRANS-SIALIDASE FROM TRYPANOSOMA CRUZI IMMUNOSTIMULATES HOST T-LYMPHOCYTES; INVOLVEMENT OF THE MAJOR LYMPHOCYTE MUCIN CD43

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It is well established that extracellular matrix proteins, such as laminin for example, act on the site specific positioning of lymphocytes. In this context, the main goal of the present work was to evaluate the simultaneous involvement of laminin and its receptor VLA6, in the myocardium T cell autoreactivity generated during experi-
ment of *Trypanosoma cruzi* infection. For that we used the mouse model of syngeneic heart transplantation, and local injection of CD4+ T lymphocytes derived from chronically chagasic donors. We observed that CD4+ T cells from mice chronically infected with Colombian strain of *T. cruzi* triggered graft rejection, and that treatment of the cells with anti-laminin receptor antibody prior to injection prevented graft rejection. These findings were confirmed when an anti-laminin antibody was injected adjacent to the grafts.

In a second vein, we found an enhancement of VL6 density on the CD4+ T cells to be injected, and a progressive increase in local laminin deposition, which was paralleled to the appearance of the cellular infiltrate.

Moreover, we observed that the pattern of laminin was also different, exhibiting a thinner laminin-containing network when rejection was prevented by anti-laminin antibody treatment. In this respect, it is noteworthy that the same treatment was able to modulate the deposition of laminin-binding pro-inflammatory cytokines IFN-γ and TNF-α, which is largely present along with graft rejection (including within the grafts), and that become restrictedly located in the periphery of the heart, except for some rare cytokine-positive cells within the transplanted organ.

From a conceptual point of view, our results correspond to *in vivo* evidence demonstrating the concept of ecotaxis, which takes into account the migrating cells as well as the microenvironment where the cell migrates.

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quence of the decrease production of IFN-γ and enhanced production of the cytokines IL-10 and IL-4 in response to antigen stimulation in vitro. These data are in striking accordance with a recent report showing similar results in the Fas-L deficient (gld) mice (5) infected with T. cruzi. Together, these results suggest that Fas-FasL-induced apoptosis might be important to control the hiper-responsiveness and favor an efficient immune response against T. cruzi and maybe to promote the establishment of a chronic disease.

References

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S10-1 – TELOMERASE AND TELOMERE ASSOCIATED PROTEINS IN KINETOPLASTID PARASITIC PROTOZOA

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Telomeres are specialized protein:DNA complexes that form the physical ends of eukaryotic chromosomes. In almost all organisms the telomeric DNA is formed mainly by sequences repeated in tandem such as in human and some Kinetoplastida protozoa whose telomeres consist of 5’-TTAGGG-3’ repeats (Moyzis, 1988; Fue Barker, 1998; Chiurrillo et al., 1999). The proteins associated to the telomeric DNA are found binding specifically to both the double-stranded repeat and to the single-strand G-rich overhang. In some protozoa, like T. brucei telomere environment, although yet obscure, is the place of important events such as antigenic variation and recombination.

We have first identified telomerase activity in extracts from insect form cells of three evolutionarily diverge Kinetoplastida species: Trypanosoma brucei, Leishmania major and Leishmania tarentolae. Telomerase activity was detected using two modifications of the TRAP assay described by Kim et al. (1994). The activity in T. brucei extracts was sufficiently robust to enable its detection by primer-extension or the conventional telomerase assay. It was demonstrated that enzyme processivity is low. The in vitro properties of telomerase suggest a possible templating domain sequence for the telomerase RNA (TER) of T. brucei. (Cano et al., 1999). Preliminary results using antisense modified oligonucleotides (2’-O-methyl-RNA) to the hypotetical TER template sequence inhibited T. brucei telomerase activity. In the future, this may provide the means to target parasite telomeres for the development of anti-parasitic drugs.

Knowing that telomerase and single-strand telomere-binding proteins share the same substrate specificity, they both bind to the G-rich telomeric DNA overhang, we decide to use telomerase positive extracts of T. brucei to isolate possible components of the telomerase holoenzyme or factors that could interact or specifically regulate T. brucei telomere length. We were able to identify three distinct protein:DNA complexes by gel mobility shift assay (GMSA) and UV cross-linking. One complex, called C3, exhibits high affinity to the G-strand telomere sequence and shows similar features to other single-stranded telomere ending-binding proteins previously described. It is also highly possible that complex C3 is tightly associated to an RNA component as the complex is completely eliminated by RNase or chemical nuclease treatment. The protein components of complex C3 were isolated from a 2D gel, eluted and their peptide sequences are under way.

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S10-2 – In vitro transposition and Leishmania genome analysis
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Genome analysis is undergoing a shift from mapping and sequencing efforts to approaches marked by a functional perspective. This transition is characterized by the design and development of genome-wide experimental strategies to evaluate gene function using the information and reagents generated by structural genomics. The purpose of functional genomics is to produce a background and starting point for further genetic and/or biochemical analysis.

The Leishmania Genome Project has generated extensive structural information. A complete cosmid-based physical map of the genome is available and sequencing of the smallest chromosome has been completed (http://mercury.ebi.ac.uk/parasites/leish.html).

We have developed a convenient in vitro transposition system that constitutes a useful tool for genomic studies. The transposable element mariner is a member of the mariner/Tc1 superfamily of transposons, which are found in a wide range of species. While many mariner elements are defective, the Mos1 element from Drosophila mauritiana encodes an active transposase. We have purified the Mos1 transposase and shown its ability to mediate transposition in vitro.

Sequence analyses of insertions into a variety of targets showed that mariner inserts randomly into target TAs. Mariner transposes with high efficiency (up to $10^{-3}$) even into large targets, such as cosmids. Also, cis requirements for transposition are restricted to short stretches of sequence within the transposon structure. These parameters permitted the construction of large insertional libraries and the design of modified mariner elements. We have used mariner insertion libraries to rapidly sequence the H region of L. major following a primer-island sequencing approach and also to map genes in Leishmania spp. by insertional mutagenesis.

Modified mariner elements containing reporter genes were constructed and will allow the generation of translational fusions when insertional libraries are introduced into the parasite. This strategy will identify expressed sequences and easily locate their products within the Leishmania cell. Furthermore in vitro insertions of appropriate transposons into Leishmania genes will generate ready-to-use reagents for systematic knockout approaches.

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S10-3 – Telomeres of Plasmodium falciparum: Not Just the End
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Telomeres play an essential role in a number of biological processes in eukaryotic cells. They ensure complete replication of chromosomes and are necessary for protection against and fusion. Chromosome ends of the protozoan malaria parasite Plasmodium falciparum are composed of degenerate G-rich repeats, in which GGGT-T (T/C) A is the most frequent and consist of distinct structural regions: the telomere and the polymorphic subtelomeric region. We observed a dimorphic chromatin structure in P. falciparum telomeres: a non-nucleosomal structure at the chromosome end and nucleosomal organisation further upstream. Enzymes involved in telomere replication and chromosome length maintenance are of fundamental importance for organisms such as Plasmodium with a highly proliferative life cycle. One of these enzymes is the specialised reverse transcriptase, telomerase, which has recently been identified in P. falciparum cell extracts. We developed an assay which allowed us to study the de novo synthesis of the highly variable telomere repeats added to the 3’ end of DNA oligonucleotide primers by plasmidial telomerase activity in P. falciparum bloodstage parasite cell extracts. In addition to elongating pre-existing telomere sequences, P. falciparum telomerase can also add telomere repeats onto non-telomeric 3’ ends. The efficient non-telomeric primer elongation was dependent on the presence of a G-rich cassette upstream of the 3′ terminus. Oligonucleotide primers derived from natural P. falciparum chromosome breakpoints are efficiently used as telomerase substrates. These results imply that P. falciparum telomerase contributes to chromosome maintenance and to de novo telomere formation on broken chromosomes. Reverse transcriptase inhibitors such as deoxyguanosine triphosphate (dTTP) efficiently inhibit P. falciparum telomerase activity in vitro. These data point to malaria telomerase as a new target for the development of drugs that could induce parasite cell senescence.

Genes coding for virulence factors are localised in the subtelomeric regions of P. falciparum chromosomes, in sites which are known to be highly recombinogenic. In several organisms, telomere-associated regions function as kind of adaptive domain, mediating rapid evolution of DNA sequences located in these regions. A recent study in our laboratory detected frequent ectopic recombinant between two members of the var gene family located on different chromosome ends. Ectopic recombination appears to be an important mechanism involved in the generation of diversity of the var repertoire.
S11-1 – RESEARCH TRENDS WITH ANAEROBIC AND AEROTOLERANT PROTOZOA—TRICHOMONAS VAGINALIS

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*Trichomonas vaginalis* is an ancient microaerophilic protist responsible for trichomoniasis (vaginitis), the number one, non-viral sexually transmitted disease (STD). The parasite survives in the constantly changing and adverse environment of the female urogenital tract. Research trends for this, and other, protozoa should focus on continuing to understand basic and fundamental aspects of the biology of the parasite itself and of the complex host-parasite interrelationship. Areas of emphasis include the following: **Metabolism**: Continued characterization of the unique aspects of the metabolism of parasites will yield insights into new targets and therapies. Novel pathways for protozoa may be discovered through the use of continuous flow cultivation under in vivo-like conditions (pH, nutrient limiting or excess, generation times, etc.) as compared to general batch cultures. **Drug resistance**: Research into the mechanisms of resistance concomitant with studies on metabolism is prerequisite for development of new drugs. This area of research, however, will yield information on the existence of general multidrug resistance genes versus unique pathways for resistance, such as gene amplification or inactivation of drugs resulting from direct modification. **Microbicides**: In addition to pharmacologic drug targeting resulting from studies on metabolism, the effect of known microbialic molecules (e.g., bile, defensins, etc.) must be examined. Further, it may be possible to generate monoclonal antibodies that are cidal in a complement-independent fashion, with such reagents useful during mucosal infections. **Pathogenesis**: Understanding the mechanisms of pathogenesis is fundamental to vaccine development. These studies will identify virulence factors with specific functional properties, such as parasite molecules mediating tissue tropism and/or cytoadherence, neutralizing immune effector molecules, and acquiring nutrients. The heterogeneity among infecting subpopulations must be delineated to understand the antigenic and functional complexity(ies) among parasites. **Epidemiology/surveillance**: There is a need to carefully establish relationships between disease outcomes and symptomatology with the infecting isolate types. Similar correlates with specific virulence properties and molecules is warranted. For example, isolate types may be defined on the basis of the presence or absence of a double-stranded (ds) RNA virus and satellite dsRNAs within protozoa. **Genomics**: Investigators in the future will have access to entire genomic sequences for protozoa. This post-genomics era will have sequence databases from which to make comparative analyses with other known pathogens, providing information on novel pathways of metabolism, strategies for immune evasion, molecules for tissue parasitism, and unique nutritional requirements. A holistic approach: A knowledge base that will lead to infection and disease intervention strategies will require a holistic approach to the basic biological research on pathogenic protozoa.

S11-2 – THE REVERSIBLE INTERNALIZATION OF THE FLAGELLA IN TRITRICHOMONAS FOETUS—THE PSEUDOCYST FORMATION

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When stimulated the eukaryotic cell has the ability to either change its surface topography, forming projections or depressions, or internalize significant portions of the plasma membrane through endocytic processes. Studies carried with some protozoa, such as *Tritrichomonas muris* and *Trichomonas batrachorum*, have shown that under certain conditions trophozoite forms transform into rounded motionless forms known as pseudocysts. Early morphological investigations showed that a true cyst wall was not observed in such forms, however, the undulating membrane and the flagella appeared to be internalized. In the present study using the cattle pathogenic protozoan *Tritrichomonas foetus* we established conditions to induce a reversible internalization of the flagella and analyzed this process using scanning and transmission electron microscopy, videomicroscopy, freeze-fracture, cytochemistry and several drugs such as colchicine, cytochalasine, nocodazo and starvation. In order to induce transformation two procedures were used: (1) The parasites were cooled from 37°C to 0°C and maintained at this temperature for 60 min, and then rewarmed to 16, 18, 23, 30 or 37°C. Samples were analyzed by light microscopy after 3, 20, 120 and 240 min; (2) The cells were maintained under starvation conditions in PBS for 3 hs and then examined. About 300 cells were examined and the percentage of cells showing the (a) 3 anterior and 1 recurrent flagela, (b) only the 3 anterior flagella, (c) two anterior and 1 recurrent flagella, (d) only two anterior flagella, (e) 1 anterior and 1 recurrent
flagella, (f) only 1 anterior flagellum, and (g) no exposed flagellum. Observation by light microscopy of *T. foetus* submitted to variation in temperature or starvation conditions showed that the cells become rounded and the flagella gradually disappeared. This process was further analyzed in experiments in which the cells were submitted to variation in temperature. Incubation of the protozoa at 8 or 12°C led to a significant decrease in the percentage of cells showing the three anterior flagella and the tip of the recurrent flagellum, predominating cells showing no flagella or only one anterior flagellum. This effect was dependent on the incubation time, reaching a plateau after 120 minutes. No free flagella were observed in almost all cells maintained at 0°C for 90 minutes. When the cells were first incubated for 60 minutes at 0°C and then at 16, 18, 23, 30 or 37°C the flagella gradually reappeared. This recovery process was dependent on both the temperature and the incubation time, being faster and reaching higher recovery indexes when incubation was carried out at 37°C for 120 minutes. Recovery did not occur at temperatures lower than 16°C. Reappearance of the flagellum depends on the presence of Ca^{2+} and Mg^{2+} in the incubation medium.

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**S11-4 – INTEGRINS IN TRICHOMONAS VAGINALIS**

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*Trichomonas vaginalis* is a well known parasitic protozoan which is usually found in urogenital cavities of humans. Most of women harbouring trophozoites of *T. vaginalis* present the sexually transmitted disease (STD) known as trichomoniasis or trichomonosis. Despite of such disease to be one of the five widespread STD, mainly present among young people, just now the pathogenic mechanisms of *T. vaginalis* starting to be elucidated.

*Trichomonas vaginalis* is able to recognize each one of RGD and YIGSR-containing glycoproteins. The *T. vaginalis* cell surface is a mosaic of well defined molecules including adhesins, proteases, lectins, and alpha 3 beta 1 integrins at least. The activities of each one of the components belonging the surface molecular repertoire of *T. vaginalis* is revelead itself during the responses of the parasite to the various types of extracellular *stimuli*. These *stimuli* include host cells, soluble factors, baterial flora of the vagina, etc. Thus, it is reasonable to consider that the parasite is able to define strategies by which it integrates diverse signals in the decision-making process. Further studies focussing the functionality of each one of these surface molecules during the host cell-parasite interaction process is obviously of relevance.

The extracellular matrix glycoprotein *Laminin* is recognized by *T. vaginalis*, and one of the consequences of such recognizing is a detectable activity of an unusual activity of a matrix metalloproteinase (MMP) in the parasite. Such proteinase of Mr 92.000 could also be detected in the parasite when it was pre-treated with nanomolar amounts of 12-O-tetradecanoylphorbol-13-acetate (TPA). The activity of such proteinase was inhibited when parasites were previously treated with TIMP-1 which is in turn, a well known inhibitor of MMP-9. Since MMP-9 is a member of the MMP family of endopeptidases with critical role in hydrolysis of ECM we did decide to investigate the sensitivity of such MMP activity to endoglycosidase H (endo H).

Parasites were consecutively treated or not with each one of TPA and *Laminin*-1, and Endo H. Following these treatements, they had their surfaces biotinylated. Surface biotinylation and immunoprecipitation with anti-MMP-9 antibodies revealed the presence of enzyme activity only on the cell surface of *Laminin*-1 or TPA-treated parasites.

These results clearly pointed out that the intracellular signalling starting by the binding of *Laminin*-1 to alpha 3 beta integrins in *T. vaginalis* may result in the activation of a MMP-9 proteinase at the cell surface of the parasite. Such results may help to explain how *T. vaginalis* degrades host tissues.

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S12-1 – DISTINCT IMMUNE RESPONSES DETERMINE THE DEVELOPMENT OF DIGESTIVE AND CARDIAC CLINICAL FORMS OF CHAGAS DISEASE

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The involvement of cell-mediated immunity is undoubtedly of major importance in the pathology of Chagas disease. Studies on the characterization of the inflammatory infiltrates in the chronic cardiac form have demonstrated that they are composed predominantly of small lymphocytes, macrophages, plasma cells and segmented leukocytes. Immunohistochemical studies demonstrated that the cells present in the tissues are mainly CD8+ many granzyme A+ and a few macrophages/TNFβ. Analysis of the role of the various cytokines by our group has demonstrated that secretion of IFNγ can be correlated with the severe cardiac form of Chagas disease. The opposite was observed for in vitro secretion of IL-10. These results suggest a role for IFNγ on the development of the severe pathology in Chagas disease and of IL-10 in controlling morbidity. Intracytoplasmic staining for cytokines demonstrated that IL-10 is produced mainly by macrophages/monocytes (CD14+high) whereas IFNγ is produced by CD3+ cells, both ?? and ?? In cardiac patients the majority of the CD3+ cells were IFNγ+ in contrast with indeterminate patients. In gastrointestinal clinical form of the disease a significant decrease in the absolute number of CD3+ T cells as well as in CD19+ B lymphocytes were detected. The most striking observation was an inversion of the CD4/CD8 ratio, contrasting with results from cardiac chagasic patients where the ratio of these cells is normal. A decrease of the percentage of CD4+CD28+ cells and an increase in the expression of HLA-DR on CD4+ and CD8+ cells suggest that their function may be altered by the lack of CD28 expression. Immunohistochemical analysis of esophagus and colon tissues demonstrated a predominance of CD3+ T lymphocytes, CD68+ and higher number of CD4+ cells when compared to the CD8+ population. An interesting observation was the presence of cells expressing TIA-1+. Finally, we also observed that all chagasic patients with mega presented denervation associated with inflammation, whereas patients without mega can present or not denervation, but no inflammation is observed in the latter group, suggesting a role for the inflammatory process on the development of this pathology.

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S12-2 – FUNCTIONNALLY ACTIVE MUSCARINIC ANTIBODIES IN THE CHRONIC PHASE OF CHAGAS DISEASE

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Auto-antibodies to adrenergic and muscarinic receptors have been found in IgG fractions from blood samples of chronic chagasic patients. Binding of the auto-antibodies to the receptors has been demonstrated and functional alterations in second messengers level and beat rate of cultured cardiac cells have been measured. We demonstrated that IgGs from a group of chronic chagasic patients with complex cardiac arrhythmias are functionally competent in inducing reduction in beat rate and atrial-ventricular conduction block in isolated whole rabbit hearts and that these IgGs exert a muscarinic like effect that can be blocked by atropine. Additionally, these IgGs reduce whole cell and single channel L-type calcium currents in isolated myocytes from rabbit hearts studied by the whole cell patch-clamp technique, and bind allosterically to atrial muscarinic receptors in porcine hearts. Levin and coworkers have found that many of the antibodies present in cardiomyopathic chronic chagasic patients’ sera are directed against T. cruzi P ribosomal antigens. In addition, they have demonstrated a cross-reaction between T. cruzi P0 ribosomal antigens and the adrenergic and muscarinic receptors in the heart. The targeted epitope in the cardiac membranes seems to be a stretch of negatively charged amino-acids located in the second extracellular loop of b1-adrenergic and M2-muscarinic receptors. Following this lead, we found that the functionally active antibodies characterized by us are able to block the antibody activation of the M2 muscarinic receptor. This cross-reactive mechanism seems to play a major role, since a single amino-acid substitution in one of the Tcruzi peptides tested, a 13-mer derived from the P2b family, resulted in either partial or total loss of the blocking peptide activity. Interestingly, the single amino-acid substitutions tested correspond to peptides derived from the human and Leishmania P ribosomal proteins, respectively.

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S12-3 – GLYCOINOSITOLPHOSPHOLIPIDS ISOLATED FROM TRYPSANOSOMA CRUZI STRAINS AND FROM NON-PATHOGENIC TRYPSANOSOMATIDS: UPTAKE BY HUMAN ANTIGEN-PRESENTING CELLS


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Glycoinositolphospholipids that are not linked to either protein or polysaccharide are the major cell surface glycolipids in all trypanosomatids investigated to date. In previous studies, the molecular structure of GIPLs from different strains of Trypanosoma cruzi was characterized, and classified into two series based on the substituent (ethanolamine phosphate or beta-galactofuranose) on the third mannose residue (Man3) distal to inositol (Carreira et al., 1996. Glycoconjugate J 13: 955-966). Here we investigate the endocytic pathways of GIPLs from G, Y, CL, Tulahuen, and Colombiana T. cruzi strains, plus L. samuelli and P. serpens GIPLs, by human monocytes, immature dendritic cells (iDC) and macrophages. Molecules were directly coupled to FITC and the internalization analyzed by flow cytometry after 1h of incubation at 4 and 37°C. Monocytes efficiently internalized GIPL from G>Y>CL, while iDC internalized all three glycoconjugates with similar efficiency. Macrophages clearly showed an increased uptake of Y over G and CL, and even more over Tulahuen GIPL. Interestingly, only a small percentage of monocytes and iDC was capable of internalizing GIPLs from Tulahuen and L. samuelli. We performed blockage assays with the CHO portion of G strain, and competition assays with the unlabeled molecule. Our results strongly suggest that endocytic cells express receptors that recognize the CHO portion of GIPLs. We are currently investigating the role of pattern recognition receptors (PRRs) and the class Ib antigen presenting molecule CD1 on the binding and uptake of GIPL by these cells.

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S12-4 – IMMUNOLOGICAL RECOGNITION IN CHAGAS´DISEASE

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Susceptibility factors or pathogenesis “checkpoints” that lead 30% of T. cruzi- infected patients to develop Chagas’ disease cardiomyopathy (CCC) after Trypanosoma cruzi infection, while the remaining 70% fail to do so are also largely unknown. Recently, our group identified T cell clones obtained from the heart lesion of a CCC patient crossreactively recognizing cardiac myosin, the major heart protein, and the tandemly repeated, recombinant antigen B13 from T. cruzi, which bears sequence variants; such heart-infiltrating T cells display an inflammatory, T1-type cytokine profile. Furthermore, in vitro sensitization with B13 protein primes cardiac myosin-recognizing T cell clones, supporting the concept of T cell molecular mimicry between cardiac myosin and T. cruzi B13 protein. It is known that the fine recognition of epitopes, as well as the cytokine profile, can determine whether an immune response will be protective or pathogenic. We studied the recognition of B13 protein and its epitopes, in PBMC from CCC or asymptomatic “indeterminate” patients (ASY) and normal individuals (N). B13 protein was recognized by PBMC from CCC, ASY or even N individuals bearing the following HLA class II genes: HLA-DQA1*0501/DQB1*0301 (DQ7, the most frequent HLA allele), DQA1*0501/DQB1*0201 (DQ2), DR1 and DR2, in line with HLA-peptide binding assays. Contact residues with HLA-DQ7 were the underlined central glycine and alanine residues in peptide KPPPFGQAAAGDKP. Ten 15-mer synthetic peptides containing all sequence variants in B13 protein were tested in proliferation assays among HLA-DQ7+ patients, and were differentially recognized in the CCC and ASY groups. In addition, ELISPOT assays on PBMC indicated that the frequency of cells producing IFN-γ after PHA stimulus was significantly higher in PBMC from CCC than ASY or N. Among CCC patients, the distinct recognition repertoire of B13-specific T cells, as well as the increased frequency of IFN-γ producing cells, could play a role in the pathogenesis of the disease, perhaps by influencing the generation of pathogenic, myosin cross-reactive T cells.

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S13-1 – BISPHOSPHONATE DERIVATIVES AS CHEMOTHERAPEUTIC AGENTS AGAINST TRYPA-NOΣOMA CRUZI

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We have recently reported, using high field 31P nuclear magnetic resonance spectroscopy (NMR) combined with specific enzymatic and chromatographic assays, that inorganic pyrophosphate (P₂O₇⁴⁻, PPi) is the major high energy phosphate compound in the parasitic protozoa Trypanosoma cruzi, Trypanosoma brucei, Leishmania amazonensis, and Toxoplasma gondii, being 5-15 times more abundant than ATP (18). PPi is present in all subcellular fractions, but is mostly concentrated in acidocalcisomes, specialized acidic vacuoles which constitute the largest calcium reservoir in Trypanosomatid and Apicomplexan parasites (6,8,9,15,20). New in vivo experiments in T.cruzi epimastigotes, using both 31P and 13C NMR, have demonstrated that glucose consumption is associated with large changes in the levels of PPi and nucleotides, indicating an active role of the former in the energy metabolism of this parasite. These facts are consistent with the recent discovery of several PPi-dependent enzymes in T.cruzi, including a glycosomal pyruvate, phosphate dikinase (2) and proton-pumping pyrophosphatases located in both acidocalcisomal and plasma membranes (ref.14 and Docampo et al., unpublished observations). Bisphosphonates, metabolically stable PPi analogs which have been used in humans to treat bone resorption disorders (7,10,11), block the proliferation of T.cruzi amastigotes inside cultured myoblasts with IC₅₀ of 65 µM for both pamidronate (Aredia®, Novartis) and alendronate (Fosamax®, Merck). Initial studies in a murine model of acute Chagas disease have shown that pamidronate at 10 mg/Kg.d given intravenously for 7 days can completely suppress the proliferation of T.cruzi in vivo while the drug pressure is maintained. Further studies with different treatment protocols and a chronic model of the disease are currently in progress. The mechanism of action of bisphosphonates against T.cruzi could include selective inhibition of PPi-dependent parasite enzymes such those indicated above and/or blockade of isoprenoid biosynthesis, as demonstrated for osteoclasts and Dictyostelium discoideum (1,3,4,10,12,13,19); in the latter case, synergic effects should be observed when used in combination with sterol biosynthesis inhibitors (5,16,17). Taken together, the results indicate that bisphosphonates may constitute a new chemotherapeutic approach for the specific treatment of Chagas disease.

References:
S13-2 – THE COOPERATIVE EFFECT OF THE HOST IMMUNE SYSTEM ON THE EFFICACY OF BENZNIDAZOLE THERAPY OF EXPERIMENTAL CHAGAS’ DISEASE

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The interaction of host immune system and chemotherapy against parasites has been previously reported in the treatment of African Trypanosomiasis, Leishmaniasis (Berger & Fairlamb, 1992), Schistosomiasis (Brindley & Sher, 1987, Fallon et al., 1992) and Malaria (Target, 1992). Different authors observed that the efficacy of drug treatment appears to be lower in immunosuppressed animals, demonstrating that the immune system of infected host, can play an important role in successful chemotherapy. Toledo et al. (1991) observed that cyclophosphamide-induced immunosuppression decreases the percentage of cure in mice inoculated with different T. cruzi strains and submitted to specific treatment with benznidazole (BZ). Michailowsky et al. (1998) also demonstrated that neutralization of endogenous IFNγ or IL-12 in vivo, reduces the efficacy of BZ treatment during the acute phase of experimental Chagas’ disease. In fact, it is well known that the treatment with nitroheterocyclic derivatives is more effective during the acute phase of Chagas’ disease, when a strong activation of the cellular compartment of the immune system and high levels of IFNγ, IL-12 and other pro-inflammatory cytokines are observed (Brender & Gazzinelli 1997).

To further investigate the cooperative effect of BZ treatment and macrophage activation during the experimental therapy of acute phase of Chagas’ disease, we used a previously selected BZ resistant population of T. cruzi (Murta & Romanha 1998) and its parental Y strain (susceptible). Mice infected with either parasites were treated at the peak of parasitemia with a high dose of BZ (500 mg/Kg of body weight). Trypomastigotes isolated from untreated infected mice, as well as, three hours after treatment with BZ were incubated with inflammatory macrophages and used to study phagocytosis, parasite destruction, cytokine release and reactive nitrogen intermediates (RNI) synthesis. According to previous study using the Y strain of T. cruzi (Lages-Silva et al. 1990), our results demonstrate that phagocytosis and destruction of the drug-susceptible parasites were significantly enhanced by drug treatment. These enhancements were accompanied by an increase in cytokines (IL-12 and TNFα) and RNI release by murine inflammatory macrophages primed with IFNγ. In contrast, BZ treatment of mice infected with drug-resistant T. cruzi population showed no effect whatsoever. The synthesis of IFNγ and RNI by splenocytes of mice infected with either susceptible and drug-resistant parasite populations, before and after treatment with BZ were also studied. Only the splenocytes from mice infected with the drug-susceptible parasites treated with BZ produced high levels of IFNγ and RNI. Our findings indicate that BZ acts on the drug-susceptible T. cruzi parasites by enhancing the phagocytosis and the production of cytokines and RNI, thus, favoring the destruction of the intracellular parasites by the cellular compartment of the immune system.

Comparative studies of drug susceptibility on T. cruzi, demonstrate no correlation between parasite drug susceptibility in vitro and in vivo (Scotch & Mathews 1987, Neal & Van Bueren 1988, Ribeiro-Rodrigues et al. 1995). These studies suggest the involvement of the immune system in the efficacy of treatment of T. cruzi infections. Taken together, our results suggest the existence of a cooperation between BZ treatment and the immune response against T. cruzi, which ensure the efficacy of drug therapy. Interestingly, this cooperative effect is observed on the susceptible but not on the drug-resistant T. cruzi population and may explain, in part, a mechanism by which T. cruzi parasites may resist to chemotherapy in vivo.

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References
Parasitic protozoans utilize glycosylphosphatidylinositol (GPI) molecules as the major mode of surface anchoring of proteins. We have previously addressed the critical requirement of GPIs in the development, infectivity and virulence of *T. cruzi* and *Leishmania* by heterologous expression of *T. brucei* GPI specific phospholipase C encoding cDNA. The expression of GPI-PLC in *T. cruzi* and *Leishmania* resulted in an apparent deficiency of GPIs, a reduction in the surface expression of GPI-anchored proteins and the inability to maintain replication as amastigote forms. These results have provided the impetus for the identification, cloning and characterization of the genes that are essential for amastigote replication and are the targets for designing inhibitors of chemotherapy importance.

We have used reverse genetic approaches that are based upon the presence of significant homology for the gene of interest among different organisms for the identification of a GPI8 homologue of *T. cruzi*. In addition, we are pursuing direct genetic approaches for the isolation of GPI-biosynthesis genes that do not share significant homology with yeast or human GPI-biosynthesis genes. We have devised a robust mutagenesis/screening approach for the selection of mutants that were capable of in vitro growth as epimastigotes and differentiation into infective metacyclics, but are unable to sustain replication as intracellular amastigotes the phenotype seen in GPI-PLC transfectants. A proportion of these “replication incompetent” mutants exhibit incomplete GPI-biosynthesis and the accumulation of various GPI-intermediates. The inability to make complete GPIs was associated with a reduction in the surface expression of GPI-anchored proteins GP50/55 and 1G7 in epimastigotes. These results suggest that our selection strategy has allowed us to identify *T. cruzi* “replication incompetent” mutants that are defective in the GPI-biosynthesis gene(s) function. Future studies will focus on the identification and functional characterization of the genes involved in GPI-biosynthesis in these parasitic protozoans.

**S13-4 – EFFECT OF CHEMOTHERAPY WITH BENZNIDAZOLE ON THE IMMUNE RESPONSE AND HEART INFLAMMATORY LESIONS IN MICE INFECTED BY TRYPANOSOMA CRUZI**

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Chagas’ disease is an important public health problem in Latin America and the main cause of myocardium diseases. In Brazil, only one drug with trypanocidal effect is available for clinical use: N-benzyl-2-nitroimidazole-acetamide (benznidazole, Bz). Bz shows many side effects and has restricted efficacy over different parasite strains. In this study, we analysed clinical and immunological parameters in the lymphoid organs, as well as cardiac involvement of mice infected by the Y strain (Bz-sensitive) of *Trypanosoma cruzi* and submitted to chemotherapy with Bz. Parasite load (100mg/kg of Bz per os 0-8 days post infection - dpi) or reduction schemes (0.25mg/mL of Bz in the drinking water 7-21 dpi) were compared. We did not observe any noticeable effect of Bz in non-infected animals. The classical description of experimental *T. cruzi* infection resulted in an apparent deficiency of GPIs, a reduction in the surface expression of GPI-anchored proteins GP50/55 and 1G7 in epimastigotes. These results suggest that our selection strategy has allowed us to identify *T. cruzi* “replication incompetent” mutants that are defective in the GPI-biosynthesis gene(s) function. Future studies will focus on the identification and functional characterization of the genes involved in GPI-biosynthesis in these parasitic protozoans.
splenomegaly, specially in the 14\textsuperscript{th} dpi, with a decrease of the B cells, and an increase of the CD4\textsuperscript{+} cells number, suggesting that only this latter population was proliferating \textit{in vivo}. We also observed partial reversal in the expression of activation markers in the spleen and lymphnodes, indicating that the cells remained partially activated, but did not follow the activation pathway up to apoptosis induction. We concluded that the treatment with Bz of \textit{T. cruzi}-infected animals should not be considered simply as a way of reducing parasitaemia and mortality. As Bz treatment causes alterations in the immune system and cardiac enzyme leakage that do not show correlation with the parasitic load, it is necessary a long-term experimental study about the repercussion of these alterations not only on the later stages of the disease, but also in auto-immune reactions and resistance to re-infections. If similar results could be obtained in acute human cases of \textit{T. cruzi} infection, then treatment in endemic areas with ongoing active parasite transmission should be re-evaluated.

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