BC-1
TRYPTOPHAN DEGRADATION IN HUMAN FIBROBLASTS INDUCED BY INTERFERON-g IS PARTIALLY INHIBITED BY *TRYPANOSOMA CRUZI*

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The effect of interferon-g (rIFN-g) in the control of *Toxoplasma gondii* and *Leishmania donovani* replication in cells of human origin, is due, in part, to the induction of indoleamine 2,3-dioxygenase (INDO), enzyme that degrades the essential amino acid tryptophan to kynurenine. Ceravolo e cols. (1996) showed that the treatment with rIFN-g had no effect on the replication of *T. cruzi* (Y strain) in human fibroblasts. The authors showed that rIFN-g induced significant amount of INDO mRNA in the human fibroblasts and that *T. cruzi* was not sensible to tryptophan degradation catalyzed by this enzyme. In this study we have investigated the participation of tryptophan metabolism in the resistance of *T. cruzi* to rIFN-g stimulation in human fibroblasts. The INDO activity of the cells was determinated by high-performance liquid chromatography (HPLC). The levels of tryptophan and kynurenine were analyzed in acid-soluble supernatants of fibroblasts activated by rIFN-g and infected by *T. cruzi*. Infected cells showed levels of kynurenine lower than the non infected ones. The presence of the parasites reduced in 35% the levels of kynurenine in the supernatants of the activated cells. This reduction was time-dependent with the parasite-cell contact. The addition of soluble protein extract of tissue culture trypomastigotes reduced in 47% the production of the kynurenine in cells stimulated with rIFN-g. These results suggest that the parasite may be able to produce a partial inhibition of the enzyme INDO. This inhibiton may explain part of the *T. cruzi* resistance to tryptophan degradation induced by rIFN-g in human fibroblasts.

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BC-2
TRYPANOSOMA CRUZI GENOME PROJECT: CONSTRUCTION OF METACYCLIC AND TISSUE CULTURE-DERIVED TRYPOMASTIGOTE CDNA LIBRARIES

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A joint effort of several laboratories is in progress to map and sequence the entire *Trypanosoma cruzi* genome. Complete genomic nucleotide sequences allow whole genome studies, which include large scale genome organization, sistematic knockout to determine gene function, characterization of the complete set of expressed sequences of a given cell type (sometimes called a transcriptome) and evolutionary comparisons between organisms. Genome projects are greatly aided by the sistematic, large-scale sequencing of randomly picked clones of cDNA libraries, which generates expressed sequence tags (ESTs) for the mapping effort and facilitates gene discovery. We have constructed four directionally cloned, oligo(dT)-primed cDNA libraries of poly(A)+ RNA from clone CL Brener epimastigotes: two standard, non-normalized libraries, a library enriched with abundant clones and a normalized cDNA library. The frequency of individual cDNA clones in a normalized library are brought within a narrow range, thereby greatly increasing the cost-effectiveness of large-scale sequencing. Such large-scale sequencing of the epimastigote normalized cDNA library is currently in progress in several laboratories. We have also been collecting purified metacyclic and tissue culture-derived trypomatigotes for construction of the respective directional cDNA libraries. Due to the limited and irregular efficiency of the metacyclogenesis obtained and the low yield of tissue culture-derived trypomatigotes, we decided to generate the libraries from total RNA by RT-PCR using the mini-exon and oligo(dT) as primers. Total RNA have already been extracted from both cell forms, and the cDNA libraries are currently being constructed.

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BC-3
TRYPANOSOMA BRUCEI RNA EDITING BY U INSERTION AND U DELETION IS CATALYZED BY A SEVEN POLYPEPTIDE COMPLEX

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RNa editing of mitochondrial mRNAs by specific insertion and deletion of U residues is a unique form of post-transcriptional maturation only found in *Trypanosoma, Leishmania* and other Trypanosomatid parasites. Some

mRNAs are edited at hundreds of sites by U insertion creating more than 50% of the mature sequence. Editing is directed by trans-acting gRNAs, which transfer their information via Watson-Crick and G:U non-canonical base-pairing to mRNA. Thus, U residues not hybridized to gRNA are removed, while gRNA non-hybridized purine residues (A or G) direct an equal number of Us to be inserted. The editing mechanism (1) involves a) gRNA-directed endonuclease, b) U addition by a terminal U-transferase or U removal by a U-exonuclease, and c) RNA ligase activities. Surprisingly the first step of insertion and deletion is by different endonuclease activities, and the second step is not by a reverse reaction but by distinct activities (2). Despite these biochemical differences, all editing activities reside in a seven major peptides complex that catalyses both deletion (3) and insertion (4). *In vivo* editing occur at many mRNA sites. In contrast, the currently available *in vitro* editing systems (which use synthetic RNA substrates), recreate editing at only one site. Consequently, it was not possible to study how the editing complex translocates from one site to the next in order to edit multiple mRNA sites. We have now identified relevant RNA-protein interactions between the editing complex and gRNA that have dramatic effects on editing. Based on these findings we engineered synthetic gRNAs with optimized functions, and for first time were able to recreate efficient translocation of the editing complex and processing of multiple sites *in vitro*. Also, we cloned and started the functional characterization of the editing complex polypeptides. Recombinant proteins are tested for activity and their possible effects when added back to *in vitro* editing reactions. So far two RNA ligase activities were identified, and the added back recombinant proteins show distinctive effects. These studies along with functional knock outs *in vivo* are in progress.

1 Cruz-Reyes J, Sollner-Webb B 1996. *PNAS 93*: 8901-8906.
2 Cruz-Reyes J et al. 1998. *Mol Cell 1*: 401-409.
3 Rusche LN et al. 1997. *EMBO J 16*: 4069-4081.
4 Cruz-Reyes J et al. 1998. *NAR*, in press.

BC-4
THE TRANSFECTION OF *TRYPANOSOMA CRUZI* CELL LINES WITH THE GENE OF THE CYSTEINE PROTEASE ISOFORM CRUZIPAIN 2 ENHANCES PARASITE INFECTIVITY IN HOST CELLS

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Cysteine proteases have been recently validated as therapeutical targets in *Trypanosoma cruzi* infection. Cruzipain, the major cysteine protease from *T. cruzi*, is a cathepsin L like enzyme which belongs to a polymorphic multigene family. In previous studies, we have described new cruzipain isoform which diverge more significantly from the family prototype, cruzain. Because most of the non-conserved amino acid substitutions are found in the catalytic domain of these isoforms, we predicted that cysteine protease with different kinetic properties might exist in the parasite. In the accompanying abstract, we demonstrated that recombinant cruzipain 2 (produced in *S. cerevisiae*) shows marked differences of substrate specificity and inhibitor sensitivity, as compared to cruzain. In attempt to investigate the functional role of cruzipain 2 isoform, we transfected *T. cruzi* Dm28c and X10/6 clones with the pTEX plasmid containing the intergenic region plus a full copy of the gene. Parasites were selected for resistance to 200 and 800 mg/mL of G418 and analysed for growth in LIT medium, for sensitivity to a synthetic cysteine protease inhibitor and for their rate of metacyclogenesis *in vitro*. The IC 50 for Z(Sbzl)Cys-Phe-CHN_2 was increased approximately 30 % in parasites that received the cruzipain 2 gene as compared with in the ones transfected with the plasmid alone. Metacyclic trypomastigotes obtained from transfected parasites were subsequently maintained in tissue culture for up to seven weeks. Invasion assays using Vero cells, CHO cells and primary culture of human smooth muscle showed that parasites transfected with cruzipain 2 were more infective when compared to wild type or parasites that received the plasmid alone. This phenotype was further confirmed using a *T. cruzi* cell line isolated in the laboratory which possess a low background of the major cruzipain and displays lower infectivity in mammalian cells. The transfection of this cell line with the cruzipain 2 gene was also able to restore infectivity of these parasites. The mechanisms by which this isoform contributes to parasite virulence are under investigation.

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BC-5
THE rRNA GENE PROMOTER OF TWO PHYLOGENETIC LINEAGES OF *TRYPANOSOMA CRUZI*

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Two major phylogenetic lineages of *Trypanosoma cruzi* have been defined based on rRNA and mini-exon gene sequences and RAPD analysis (Souto et al. 1996 *Mol Biochem Parasitol 83*: 141-152). We are presently studying

the structure and function of the rRNA promoter regions of the two lineages. For this purpose we selected as representatives of lineages 1 and 2, respectively, clones CL Brener and Dm28c. Sequence alignment of the rRNA promoter regions of CL and Dm28 strains showed only 82% identity, reinforcing lineage division. The transcription start point was mapped in the two *T. cruzi* clones and localized at 1800 nt from the 5' end of the 18S rRNA gene. A plasmid construct bearing CL strain promoter sequence upstream from bacterial chloramphenicol acetyltransferase (CAT) was electroporated in CL Brener and Dm28c epimatigotes. Expression of CAT was only observed in CL Brener. On the other hand, the analogous construct bearing lineage 2 promoter sequence (approximately 800 bp) was functional in both isolates. Interestingly the expression in CL Brener was 150-fold higher than that observed in Dm28c. The electroporation efficiency was analysed by Southern blot experiments and indicated that the plasmid construct was interiorized in Dm28c only 1.7-fold less in relation to CL Brener. In order to better characterize lineage 2 promoter, constructs were generated containing different portions of the promoter region. The experiments allowed to define the smallest region that efficiently drove CAT expression. Inhibitory sequences which could explain the low expression of CAT in Dm28c were not detected. The evaluation of the *trans*-splicing process of CAT mRNA in both strains is under study.

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BC-6
THE MINI-EXON GENE MULTIPLEX PCR ASSAY: A MOLECULAR TOOL TO DETECT GENETIC VARIABILITY IN HUMAN NEW WORLD TRYPANOSOMES

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Population genetics studies performed on *Trypanosoma cruzi*, using six enzyme electrophoresis profiles revealed substantial variability among isolates defining three different zymodemes. Zymodeme I and III were associated with sylvatic mammals and zymodeme II was linked mostly to human cases of Chagas disease. Extending the number of studied gene loci to 15, Tibayrenc et al. increased the number of natural clusters to 43. This extreme variability was also evidenced by several other molecular methods, such as restriction fragment polymorphism of kinetoplast DNA, DNA fingerprinting, gene localization in chromosomal blots and analysis of randomly amplified polymorphic. However, Souto et al., in 1996, studying two distinct gene loci, the ribosomal RNA gene and the mini-exon non-transcribed spacer defined a clear dimorphism (two lineages) in the taxon *T. cruzi*. Subsequently, Fernandes et al. in 1998 proposed an association of each of the lineages to the zymodemes I and II originally described by Miles and consequently to the domestic and sylvatic environments. Recently, epidemiological studies in the Brazilian Amazon isolated some *T. cruzi* stocks from *R. brethesi* that could not be typed as either lineage 1 or 2. After PCR amplification of the mini-exon repeat from these isolates, cloning of the ampified product and DNA sequencing, an additional variable region was found in the non-transcribed spacer, corresponding to an insertion of approximately 50 bp. Further experiments revealed some isolates that are considered as zymodeme III prototypes have this same insertion, indicating that the insertion can be used as a molecular marker for zymodeme III. A multiplex PCR using four sets of primers was developed in order to perform a typing assay that could distinguish among all the three different *T. cruzi* mini-exon genotypes and also *T. rangeli*. This PCR yields products of distinct sizes for each group of parasites that are easily evidenced by agarose gel electrophoresis, eliminating the need for further hybridization experiments.

BC-7
TRYPANOSOMA CRUZI RESISTANCE TO SYNTHETIC INHIBITORS OF CYSTEINE PROTEINASES CORRELATES WITH CHANGES IN THE EXPRESSION OF TARGET ENZYMES AND MEMBRANE PERMEABILITY

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Recently validated as a therapeutical target, the cathepsin L-like major cysteine proteinase of *T. cruzi* accounts for the bulk of proteolytic activity in the parasite's endolysosomal system. In light of the clonal heterogeneity and genetic diversity of *T. .cruzi*, it seemed plausible that selective pressure with CP inhibitors could favor the emergence of drug-resistant organisms. Here this problem was approached by subjecting epimastigotes (Dm28 strain) to long term exposure to a membrane-permeable peptidyl diazomethane inhibitor (Z-(SBz)cys-phe-CHN2) which preferentially inactivate cathepsin L-like enzymes, such as cruzipain. A cell line (R-Dm28) displaying stable phenotypic resistance for Z-(SBz)cys-phe-CHN2 was obtained, the Ic50 (20mM) being 13X higher than the parent strain. No cross resistance was observed with benzonidazol, an related drug. Ultrastructural analysis of R-Dm28

revealed grossly enlarge reservosomes, but the parasites could still transform into metacyclics Trypomastigotes and infect mammalian cells in vitro. Interestingly, the antigenic contents of mature cruzipain were sharply reduced in R-Dm28. We then used Biotin-LVG-CHN2, an irreversible inhibitor based on Cystatin C to identify the enzymatically active CP's present in extracts from R-Dm28. This experiments revealed that the 60Kda cruzipain enzyme of R-Dm28 was expressed in lower amounts, as compared to the parent Dm28 cell line. R-Dm28 showed decreased labelling of the 60 kDa cruzipain band. Conversely, we observed an increased reactivity with an E64 sensitive CP of 30kDa. Interestingly, this protease was also present, albeit in fairly lower proportions, in the parent Dm28 strain. The possibility that the 30 kDa CP consisted of cruzipain's cleaved central domain was ruled out because (i) the enzyme was relatively refractory to inhibition by Z-(SBz)cys-phe-CHN2 (ii) mAb to the 30 kDa protein failed to recognize cruzipain (iii) its N-terminal sequence (LQDRFDE) was almost identical to that recently reported for a cathepsin B-like enzyme of *T.cruzi*. Hence, our data suggest that the increased expression/accumulation of cathepsin B-like enzymes may partly compensate for the reduced availability of the cathepsin L-like cruzipain target in R-Dm28. The combination of these biochemical changes may have concurred to development of the resistance to synthetic CP inhibitors.

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BC-8
TRYPANOSOMA CRUZI INHIBITOR OF CYSTEINE PROTEASES: BIOCHEMICAL CHARACTERIZATION OF NATURAL FORM OF CHAGASIN

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In view of the broad distribution of cystatin-like inhibitors in nature, we set out to identify related molecules in the *Trypanosoma cruzi* species. Here, we will summarize information accumulated on chagasin, a potent reversible inhibitor of papain-like cysteine proteases (CPs), which cDNA gene was recently cloned and expressed in *Escherichia coli*. Northern blot analysis revealed that chagasin steady-state RNA levels vary during *T. cruzi* development (trypo > ama > epi). Detergent lysates from each developmental stage of *T. cruzi* were analyzed by Western blotting using an antisera produced against recombinant chagasin (r-chagasin). The data confirmed that natural chagasin (n-chagasin) had the expected molecular weight (~11 kDa) and is expressed in all three developmental stages, results being consistent with the Northern blotting data. Immunolocalization studies performed by E.M. revealed the presence of n-chagasin protein in intracellular vesicles, flagellar pocket, flagellum and surface membrane (trypo and ama). Phase partition analysis with Triton X-114 indicated that most chagasin molecules accumulate in the detergent phase of trypo and ama. In both cases, treatment with PI-PLC has completely converted membrane bound forms of chagasin into soluble forms, suggesting that GPI-anchors may be directly or indirectly responsible for chagasin association to cell membranes.

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BC-9
RNA EXPRESSION IN *TRYPANOSOMA CRUZI*: 3'UTR CIS-ELEMENT AND A PUTATIVE RNA BINDING PROTEIN POST-TRANSCRIPTIONALLY REGULATE THE AMASTIN/TUZIN GENE CLUSTER

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The genome of *Trypanosoma cruzi* contains tandemly arrayed alternating genes encoding the surface protein amastin and the G-like protein tuzin. These genes have been used as a model to study gene expression regulation during the *T. cruzi* life cycle. Our previous work has shown that the steady state level of amastin mRNA is 68-fold greater in amastigotes than in epimastigotes. In contrast, tuzin mRNA abundance is roughly equivalent in the different life cycle stages. Nuclear run on experiments have demonstrated that amastin and tuzin genes are transcribed to a equal extent in both developmental stages.

Three sets of experiments, (*i*) transient transfections with a reporter gene flanked by sequences in the 630 nt amastin 3'UTR, (*ii*) studies on the effect of cycloheximide on tuzin and amastin mRNA abundance and (*iii*) RNAse protection assays, indicate that the post-transcriptional regulation of the amastin/tuzin gene cluster involves a multi-component system of both positive and negative regulatory *cis*-elements within the 3'UTR and potentially a labile amastigote-specific *trans*-acting factor(s) that interacts with the 3'UTR. Furthemore, measuremnts of the amastin mRNA half-life in amastigotes and epimastigotes suggest that the different steady state levels of amastin mRNA are partially determined by its stability.

Using linker scanning mutagenesis of the amastin 3'UTR, we have identified a 70 bp sequence within the 630 UTR that is critical for the up regulation of a reporter gene in transiently transfected amastigotes. A different 70 bp region appears to confer negative regulation to the reporter gene in epimastigotes. Our current studies are directed at determining if the defined regulatory UTR *cis*-elements confer regulation by enhancing RNA stabilization or degradation, and if these *cis*-elements interact with the *trans*-acting factor seen in our RNA protection and mobility shift assays.

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BC-10
REQUIREMENT OF P38-MITOGEN ACTIVATING PROTEIN KINASE ACTIVATION FOR INDUCTION OF MONOKINE SYNTHESIS BY MURINE INFLAMMATORY MACROPHAGES EXPOSED TO GLYCOSYLPHOSPHATIDYLINOSITOL ANCHORS PURIFIED FROM *TRYPANOSOMA CRUZI* TRYPOMASTIGOTES

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The present study reports our efforts in defining the involvement of MAP kinase pathway(s) on induction of monokine synthesis by macrophages exposed to glycosylphosphatidylinositol (GPI) anchors purified from *Trypanosoma cruzi* trypomastigotes. As positive control we used LPS derived from *Escherichia coli*, known as potent inducer of different MAP kinase pathways. Among different inhibitors for protein kinases we found that SB203580, a pyridinil-imidazole compound and specific inhibitor for p38 MAP kinase, is a potent inhibitor of TNF-a and in a less extend of IL-12(p40) synthesis by inflammatory macrophages exposed to either GPI-anchored mucin like glycoproteins (tGPI-mucins) or purified GPI (tGPI) anchors isolated from *T. cruzi* trypomastigotes. In contrast, PD098059 and inhibitor of MAPKAP-K1 (MAPK-1 pathway) had no inhibitory effect on IL-12(p40) or TNF-a synthesis by macrophages expose to different microbial stimuli. We also measured the activity of downstream targets of p38 MAP kinase and MAPKAP-1 before and after macrophage stimulation with either tGPI-mucins or tGPI. Our results show that tGPI activates both MAPKAP-K1 and MAPKAP-K2. However only the MAPKAP-k2 activity was inhibited by the compound SB203580. The peak of MAPKAP-K2 activity induced by tGPI was at 15 min post macrophage simulation. We also studied the ability of different cytokines (i.e. IFN-g and TNF-a), which are known to potentiate monokine synthesis by macrophages exposed to different microbial products, on their capacity to induce p38 MAP kinase activation. Despite of previous studies showing that TNF-a activates the p38 MAP kinase, in our system TNF-a had no effect in inducing or potentiating MAPKAP-K2 activity on macrophage lysates. In contrast, IFN-g a potent enhancer of IL-12(p40) and TNF-a synthesis by macrophages stimulated with microbial products was shown to significantly augment the activity of MAPKAP-K2 induced by either tGPI or LPS. Consistent with these findings, we observed that lysates obtained from macrophages derived of Interferon Consensus Supressor Binding Protein knockout mice, known to be defective in their IFN-g priming for IL-12(p40) synthesis, express less MAPKAP-K2 upon stimulation with tGPI or LPS in the presence of IFN-g. Finally, we studied the ability of different compounds (i.e. cAMP analogues, dexametazone and IL-10) known to be potent inhibitors of IL-12(p40) and TNF-a synthesis in their ability to inhibit MAPKAP-K2 activation induced by tGPI or LPS in the presence or absence of IFN-g. Our results show that cAMP analogues and IL-10 had no modulatory effect on MAPKAP-K2 activity present in lysates from macrophages stimulated with tGPI/LPS and/or IFN-g. In contrast, dexametazone had a partial but consistent inhibitory effect on MAPKAP-K2 activity elicited by the different microbial stimuli. These studies indicate the involvement of p38 MAP kinase pathway on the induction of TNF-a and IL-12 synthesis by macrophages exposed to GPI anchors derived from *T. cruzi* trypomastigotes.

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BC-11
REDUCTION IN CARDIAC MYOCYTE CALCIUM CURRENTS BY IGGS FROM CHRONIC CHAGASIC PATIENTS, CAN BE MEDIATED BY a-ADRENERGIC RECEPTOR

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Interactions between sera of chronic chagasic patients and muscarinic or b- adrenergic receptor has been demonstrated by several laboratories. These interactions may be due to molecular mimicry between parasite ribosomal protein and the second extracelular loop of G-protein coupled receptors. Recently Farias de Oliveira et al., demonstrated that sera from chronic Chagasic patients (ChrCh), can induce conduction block and/or decrease heart rate in

isolated rabbit heart and this effect was abolished by atropine. However this muscarinic-like effect can not be blocked by atropine in 53% of all sera tested. We therefore decided to investigate the interaction of CrChr sera with a- adrenergic receptor using the Langerdoff and path clamp technique. ECG was continuously monitored in isolated rabbit hearts during perfusion with Tyrode solution containing (mM) NaCl 150.8; MgCl 0.5; Glucose 6.0; $NaHCO_3$ 18 mM; $CaCl_2$ 2.7. equilibrated with a mixture of 95% O_2 and 5 % CO_2. All tested sera induced AV block in presence of atropine. After the control record, sera of ChChr (1:100; v:v) in presence of Yoimbine (10^{-9}M) (a-adrenergic receptor antagonist) was added to the Tyrode solution The calcium current was recorded in dissociated ventricular myocytes from rabbit hearts and analyzed than by whole cell path clamp technique. To isolate the calcium current we used a pipette solution containing (mM) CsCl 120; NaCl 20; $CaCl_2$ 0.5; HEPES 10; TEA - Cl 20. After control records we tested the effect of purified IgG, from ChChr (30mg/ml) in presence of Yoimbine. Four out of 8 non-muscarinc sera tested in isolated heart had their effect blocked by Yoimbine. All three IgG purified from non-muscarinic sera had the effect blocked by Yoimbine in isolated myocytes, while this IgG 's induced a significant decrease in calcium currents in the absence of a adrenergic receptor blocker. We conclude that IgG's, present in the serum of ChChr, may reduce the calcium current by activation of a- adrenergic receptors.

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BC-12
RECOMBINANT CYSTEINE PROTEASE ISOFORMS FROM *TRYPANOSOMA CRUZI* (CRUZAIN AND CRUZIPAIN 2) SHOW MARKED DIFFERENCES TO INHIBITOR SENSITIVITY

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It is believed that cysteine proteases are essential for *Trypanosoma cruzi* survival, since their inhibition blocks parasite development in the mammalian cell. Cruzipain (or cruzain), the major cysteine protease of *T. cruzi*, belongs to a polymorphic, multigene family. We have previously identified a new cruzipain isoform (cruzipain 2) which diverges more significantly from the family prototype, cruzain (cathepsin L-like enzyme). Since most of the non-conservative amino acid substitutions in cruzipain 2 are found in the catalytic domain, we predicted that this isoform may display kinetic properties different from those of cruzain. Here we confirm that recombinant cruzipain 2, produced in *S. cerevisiae*, possesses different substrate specificity and pH stability as compared to r-cruzain. The existence of cruzipain isoforms with markedly different kinetic properties may have obvious implications on the development of trypanocidal cysteine protease inhibitors. In order to test this possibility, the sensitivity of both recombinant isoforms to synthetic and natural inhibitors was compared. The data show that cruzipain 2 is 30 times less sensitive to E-64 and 500 times less sensitive to human high molecular weight kininogen than r-cruzain (a gift from Dr J McKerrow). However, both isoforms displayed similar inhibition constants to rat T kininogen and chicken cystatin (only 3 fold higher with cruzipain 2). The functional role of cruzipain 2 is being currently investigated.

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BC-13
r-CHAGASIN, A POTENT INHIBITOR OF PAPAIN-LIKE CYSTEINE PROTEINASES, REDUCES HOST CELL SUSCEPTIBILITY TO INVASION BY *TRYPANOSOMA CRUZI* TRYPOMASTYIGOTES

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In spite of the progress obtained in the biochemical and structural characterization of natural inhibitors of papain-like cysteine proteinases (CP), their precise role in cellular homeostasis is poorly defined. The present study was motivated by the recent characterization of chagasin, a novel class of potent inhibitors of papain-like CP's, recently identified in *Trypanosoma cruzi* organisms. As mentioned in an accompanying abstract (Monteiro et al., this issue), this protein is expressed in relatively high amounts in tissue culture trypomastigotes (TCT), and its cytochemical distribution is compatible with extracellular function(s). We reasoned that chagasin may encounter host cell lysosomal CP's during the brief period which *T. cruzi* trypomastigotes stay in the parasitophorous vacuole. This possibility seemed worth investigating, in light of recent evidences showing that *T. cruzi* actively stimulates lysosomal exocytosis in fibroblasts and epithelial cells, in early stages of the invasion process (Rodriguez et al. 1997 *JCB 1*: 93-104). Accordingly, the directional movement of lysosomes towards sites of parasite attachment is

brought about by successive intracellular free Ca+2 transients induced by the parasite. Interestingly, this exocytic pathway probably corresponds to a previously described repair system responsible for membrane resealing in wounded cells. It is thus possible that *T. cruzi* has taken advantage of Ca+2-regulated membrane repair mechanisms to penetrate in at least some cell types. It is also thought that the acid pH of the parasitophorous vacuole is sustained by discharges of lysosomal contents. Luminal acidification optimizes the TS-dependent desialylation of membrane-bound vacuolar glycoproteins, thus setting conditions for membrane-disruption by Tc-Tox. Although host lysosomal enzymes (for example, the papain-like cathepsins B,L,H,S) are presumably released into such vacuoles, nothing is known about their role(s), if any, in the host-parasite relationship. Here we present preliminary data obtained in vitro, showing that the endocytic uptake of recombinant chagasin leads to a marked reduction of the intracellular activity of lysosomal CP's. Intriguingly, these biochemical effects were accompanied by an effective reduction of host cell invasion by Dm28 trypomastigotes (Vero and CHO cell lines).The data suggest that the activity of host lysosomal CP's somehow enhances the invasion process. The possibility that natural CP inhibitors (such as pathogen-derived chagasin) may indirectly regulate the lysosomal exocytic pathway of mammalian cells is being presently investigated.

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BC-14
rASP1 RECOGNITION BY SERA OF PACIENTS INFECTED WITH *TRYPANOSOMA CRUZI*

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The obligate intracellular protozoan parasite, *Trypanosoma cruzi*, is the etiologic agent of Chagas' disease. In contrast to the trypomastigote and epimastigote stages of *T. cruzi*, studies involving the intracellular amastigote stage of the parasite have been limited, particularly in relation to amastigote surface molecules. To better understand this stage of the parasite, we screened an amastigote cDNA expression library, resulting in the isolation of the gene that encodes an 80 kDa amastigote surface protein (ASP-1), a member of the *T. cruzi* trans-sialidase superfamily. Studies using ASP-1 derived peptides identified a CTL epitope on the basis of its ability to bind to class I MHC molecules and to sensitize cells for lysis by CTLs obtained from *T. cruzi*-infected mice. To further characterize ASP1, we subcloned the gene into an expression vector, pET32a, transformed *E. coli*, AD494, and induced the rASP1 expression with IPTG, resulting in an overexpression of rASP1. Since, only recombinant proteins derived from epimastigote and trypomastigote stages of *T. cruzi* have been applied in the sorodiagnosis of Chagas disease, we decided to evaluate rASP1 as a potential candidate to the diagnosis of chagasic patients. Preliminary results, using lisate of IPTG-induced recombinant bacteria, showed that 82% of the chagasic sera tested by Western Blot had antibodies against rASP1. At present, we are purifying the recombinant protein and developing an ELISA Assay to determine the actual sensitivity and specificity of rASP1.

BC-15
PURIFICATION AND KINETIC CHARACTERIZATION OF TWO ENZIMES MALATE DEHIDROGENASE PRESENT IN *TRYPANOSOMA CRUZI*

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Two malate dehidrogenases (MDH) activities have previously been reported in *Trypanosoma cruzi:* one of them apparently belonging to the Krebs cycle and located in the mitochondrion and the other one in the glycosome, a peroxisome like organelle, as are all the trypanosomatids, of transforming glucose into 3-phosphoglicerate (or 1,3-diphosphoglicerate). MDHg has been responsabilized for the reoxidation of the NADH produced during glycolisis by glyceraldehide phosphate dehidrogenase. Both counterparts were purified, MDHm from the soluble fraction by diferential centrifugation after silicon carbide grinding and MDHg from purified glycosomes. Both enzymes were purified up to apparent homogeneity, according to SDS-polyacrylamide gel electrophoresis, which corresponded to 149 fold for MDHg and 421 fold for MDHm. Detailed kinetic studies were performed on both counterparts: Km for oxalacetate were about 40mM and 110mM and for NADH about 100mM y 40mM respectively for MDH_g and MDH_m, indicating slight differences in the apparent affinities of both enzymes for their substrates. We were not able to determine kinetic parameter in the malate-oxalacetate direction, the normal direction in the Krebs cycle. Sensitivity to ionic strength was different in both enzymes with 130mM optimum for MDH_g and 50mM for MDH_m. Optimum pHs were also different; more acidic for MDH_g. The molecular weight of both subunits were determined by SDS-PAGE, indicating a small but consistent difference between both counterparts: 33 kDa for MDH_m and 35 kDa for MDH_g. In both cases of native enzyme appeared as homotretamers according to their migrations on sephadex G-100: 132 kDa for MDH_m and 135 kDa for MDH_g.

BC-16
PRIMING WITH SELF-HEART ANTIGENS TRIGGERS MYOCARDITIS IN MICE INFECTED WITH A LOW VIRULENCE STRAIN OF *TRYPANOSOMA CRUZI*

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Infection with *Trypanosoma cruzi* elicits inflammatory cell accumulation in the heart and often leads to severe myocarditis. The pathogenesis of heart damage is controversial and autoimmune phenomena have been implicated in the etiology of the chronic cardiopathy of Chagas' disease. In this study we have investigated the effect of immunization with crude heart extracts on the development of myocarditis upon infection with *T. cruzi*. DBA/2 or C57Bl/6 mice were repeatedly immunized with heart extract/complete Freund's adjuvant - CFA (Group 1) or saline/CFA (Group 2) for 2 months. After complete immunization protocol the animals were bled and sacrificed for histopathological analysis. No pathological changes were observed in myocardium of either group. *In vitro* proliferative response of spleen cells to myocardial antigens was undetectable in both experimental groups in spite of detection of anti-heart antibodies in group 1 (ELISA). Mice were challenged i.p. with 10^2 blood trypomastigotes (Y strain). In this model, low numbers of parasites were detectable in the bloodstream up to day 30^{th} after infection and no mortality was observed. After 60 days of infection, group 1 mice infected with *T. cruzi* developed intense myocarditis as judged by gross alterations (cardiac enlargement plus apical aneurysm) and histopathologic changes (multifocal diffuse myocarditis). The severity of lesions showed a clear correlation with the number of doses employed for immunization. In contrast, infected animals from group 2 showed only mild inflammatory changes of heart muscle. Skeletal muscle appeared normal in both groups. These results suggest that immunization with heart extract leads to expansion of heart-specific self-reactive lymphocytes that promote myocarditis albeit heart fibers are damaged by *T. cruzi* infection.

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BC-17
PRELIMINARY BIOCHEMICAL ANALYSIS OF A PURIFIED PARAFLAGELLAR ROD FRACTION

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The flagellum of tripanosomatid protozoa possesses, apart from the common 9+2 microtubular axoneme, a filamentous lattice often referred to as paraflagellar rod. This highly-ordered structure of still uncertain function runs, alongside the axoneme, throughout most of the lenght of the flagellum. The major ultrastructural features and biochemical components of the paraflagellar rod have been described in several species of trypanosomatids and are largely conserved among different members of the family (reviewed by Bastin et al. 1996 *Parasitol Today 12*: 302-307). Little is known, however, on minor biochemical components of the paraflagellar rod, although its complex ultrastructure suggests the existence of numerous such components. An interesting approach for the identification of minor paraflagellar rod proteins is the production of purified fractions of this structure.

In our study, a purified paraflagellar rod fraction of the tripanosomatid *Herpetomonas megaseliae*, non-pathogenic to humans, was submitted to SDS-PAGE and Western blot analyses. The purified fraction was prepared by a combination of conventional cell-fractionation procedures, non-ionic detergent treatment and a very brief, and highly controlled, limited proteolysis. Comparative analysis of different purification steps indicates that the purified fraction is highly enriched in the major paraflagellar rod proteins (60-62kDa), as identified by immunoblotting with the monoclonal antibody 1B10 (kindly provided by prof. Renato Mortara, USP), (Balanco et al. *Parasitology* 116: 103). Apart from the major components of the paraflagellar rod, minor protein bands (in the molecular weigh range from 70 to 200 kDa) are also enriched in the purified fraction, probably corresponding to minor paraflagellar rod components. Also, a band of 225 kDa is highly affected by the proteolitic treatment that detaches the paraflagellar rod from the axoneme and might, therefore, represent a component of the bridges that link the axoneme to the paraflagellar rod. In fact, a spectrin-like protein of similar molecular weight has been located to these bridges in *Trypanosoma brucei* (Hemphill et al. *J Struct Biol 107*: 211-220).

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BC-18
NUTRITIONAL STRESS REGULATES ADHESION TO SUBSTRATE AND METACYCLOGENESIS IN *TRYPANOSOMA CRUZI*

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Metacyclogenesis in *Trypanosoma cruzi* occurs naturally at the rectal gland of the insect vector, where epimastigotes adhere to the intestinal surface before differentiating into the trypomastigotes. Adhesion and metacyclogenesis can be induced *in vitro* by incubating epimastigotes in TAU3AAG medium, where the cells adhere to the culture flask walls. Reservosomes are endosomal compartments in epimastigotes where proteins and lipids are accumulated, being absent in cell culture derived amastigotes and trypomastigotes. It has been suggested that nutrients accumulated in reservosomes are used as energy source during cell differentiation in *T. cruzi.*

We have investigate metacyclogenesis *in vitro*, by using TAU3AAG medium supplemented with different nutrients (10% fetal bovine serum of gold-labelled transferrin) in order to analyse the relationship among nutritional stress, adhesion, endocytosis of nutrients and cell differentiation. In some experiments, the cells were cultivated for 96 hr in TAU3AAG and then the supernatant was removed and incubated with transferrin-gold complex solution. The remaining adhered cells (epimastigotes) were cultivated for 48 hr with the tracer. As a control, the supernatant was removed and transferrin-free TAU3AAG was added to the adhered cells. After each experimental schedule the parasites were processed for transmission electron microscopy. Addition of serum to TAU3AAG medium inhibited metacyclogenesis and promoted growth of epimastigotes. ultrastructural analysis of the cells showed the presence of reservosomes at their posterior end. Few cells could be observed attached to the substrate. The same result was observed when serum was added after 96 hr of cultivation. After this time, approximately 50% of supernatant cells were under the epimastigote form. Gold-labeled transferrin was found in both adhered and free-swimming epimastigote forms, but not in the trypomastigote, at any time of cultivation. addition of transferrin-gold after 96 hr of cultivation decrease the metacyclogenesis rate and the adhesion. Our results suggest that in *T. cruzi* adhesion to substrate is triggered by nutritional stress. suplementation of TAU3AAG medium with some nutrients inhibited and reverted the metacyclogenesis process and supported epimastigote growth. Our data suggest a close relationship between uptake of nutrients, adhesion and cell differentiation in *T. cruzi.*

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BC-19
MURINE IMMUNE RESPONSE AGAINST A RECOMBINANT *TRYPANOSOMA CRUZI* ANTIGEN

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The search for recombinant antigens useful for the serodiagnosis of parasitic diseases has been the aim of many research laboratories. Moreover, antigens that are able to stimulate the production of antibodies and/or that may elicit a cellular response in experimental animals are also candidates for the development of vaccines. In this work we describe the humoral and cellular immune response in mice against a recombinant *Trypanosoma cruzi* polypeptide, which was shown to be specifically recognized by serum antibodies from Chagas' disease patients (Queiroz et al. 1996 *Braz J Genetics 19*: 194). Immunoglobulin isotypes were assessed by ELISA. Mice immunized against a sonicate of *Escherichia coli*, expressing the recombinant antigen, showed a 50% increase in IgA antibodies directed against the antigen than those immunized with the wild type strain. An increase in IgA-producing B cells was also observed by ESA (ELISA Spot Assay). The IgG isotype, but not the IgM, was also increased in mice immunized with recombinant antigen, although less strongly than IgA. The antigen was immunolocalized by confocal fluorescence microscopy in the cytosol as diffuse labeling, with absence of staining over nucleus, kinetoplast, flagellar pocket and flagellum. Preliminary sequence data of the cDNA clone showed a discrete homology with P ribosomal protein and no homology to other common *T. cruzi* antigens as CRA, FRA or trans-sialidases. Our results suggest that this antigen may be a novel candidate for the development of a vaccine.

BC-20
MOLECULAR CLONING OF A *TRYPANOSOMA CRUZI* CLATHRIN HEAVY CHAIN GENE

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Endocytosis is an essential mechanism to maintain cellular homeostasis and formation of coated vesicles characterize an endocytosis specialized in small molecules transport. This coat derives from citosolic heteroligomeric complexes assembly and one of the best characterized coat complex has clathrin as the major constituent. Clathrin consists of three heavy chains (190 kDa) associated with three light chains (30 kDa) and it plays structural role in deforming the underlying membrane to form the budding vesicle. In trypanosomatids, the endocytosis process is not well understood, but it is known that the flagellar pocket membrane is an obligatory intermediary station for membrane-bound molecules trafficking. Here, we report the *Trypanosoma cruzi* heavy chain clathrin gene cloning and its characterization. About 8.0 kb were sequenced: the open reading frame is 5.2 kb long and the remaining sequence represents the 5' and 3' flanking regions. The northern blot analysis showed a 7.3 kb transcript in Dm28c epimastigote and trypomastigote forms. Besides, the Southern blot analysis indicates the presence of only one copy of that gene in *T. cruzi* genome. Sequence comparison of the predicted protein sequence showed 34 to 40% identity and 53 to 61% similarity with other characterized eukaryotic clathrin heavy chains.

BC-21
MOLECULAR CLONING AND CHARACTERIZATION OF A NEW DNAJ-LIKE PROTEIN OF *TRYPANOSOMA CRUZI*

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In eukaryotes, DnaJ-like proteins belong to the conserved heat shock protein 40 (hsp40) family and are involved in regulation of the molecular chaperones hsp70 (dnaK-like protein) mediating the biogenesis of proteins. The basic mechanism of action of the hsp70s is the ability to bind and release polypeptides which are in non-native conformations in a ATP-dependent manner (protein folding).; the subcellular compartmentalization of different hsp70 members and their specific interaction with different DnaJ-like proteins have allowed these chaperones to participate in a variety of protein biogenesis pathways including assembly and disassembly of protein complex, proteolysis, translocation of proteins into organelles and translation initiation. In *T. cruzi*, the DnaJ-like family consists of 5 members (tcj1-4 and TcDJ1) sharing a highly conserved N-terminal sequence known as J domain which allows the association with hsp70 proteins. Herein, we report a nucleotide sequence of a gene encoding a novel *T. cruzi* DnaJ-like protein which we have isolated serendipitously in a *T. cruzi* genomic DNA library. An open reading frame of 1014bp encoding a 338 amino acid protein was detected. Northern blot analysis and RTPCR confirm the expression of this gene in *T. cruzi*. Surprisingly, this novel DnaJ-like protein displays a sequence homology of 50% with a cytosolic DnaJ-like member of *S. cerevisiae*, SIS1 involved in translation initiation, whereas it displays only 25-39 % of sequence conservation with other members of the *T. cruzi* DnaJ-like family. Related to this observation, the hydrophilic character of this DnaJ-like protein and the absence of both a detectable N-terminal putative petide signal and a C-terminal CAAX motif (substrate for prenyl modification of some DnaJ-like proteins) suggest that this protein is probably cytosolic. Moreover, both yeast SIS1 protein and new *T. cruzi* DnaJ-like protein share common a J domain followed by a glycin-rich region and they lack the CRR repeat region involved in the substrate binding of some DnaJ-like proteins, hence suggesting that the SIS1 protein and this new DnaJ-like protein might have a related function. Finally, in contrast to the other members of *T. cruzi* DnaJ-like family which are encoded by single copy genes, the novel DnaJ-like protein is encoded by multicopy genes spread into the genome of *T. cruzi*.

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BC-22
L-ARGININE UPTAKE AND L-PHOSPHOARGININE SYNTHESIS IN *TRYPANOSOMA CRUZI*

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Some of the characteristics of L-arginine uptake by *Trypanosoma cruzi* epimastigotes were evaluated. Under standard conditions this is a saturable and dependent on L-arginine concentration process. Maximum velocity and km were 48.1 pmol.min^{-1} per 3x10^{7} cells and 4.16 mM, respectively. The calculated activation energy and Q10 were

about 31.1 KJ.mol^{-1}, and 6.7, respectively. Starvation time raised the uptake velocity. Cells retained the labeled aminoacid independently of the presence or absence of exogenous L-arginine.

The specificity of L-arginine uptake could be demonstrated by competition assays with 80 fold molar excess of anionic, neutral or catonic amino acids and several L-arginine derivatives. L-homoarginine, D-arginine, L-canavanine, L-ornithine and L-citrulline produced the highest inhibition. L-arginine uptake by *T. cruzi* epimastigotes was affected by low concentrations of sodium azide and dinitrophenol, while the absence of potassium or sodium ions in the incubation mixture had no effect. Uptake was also partially blocked by the sulfhydryl alkylating agent N-ethylmaleimide and no differences in the uptake rate could be detected in the pH range between 5.5 and 8.5.

The major product of the uptaked L-arginine (27.1%) was characterized as phosphoarginine and the 4.3% was incorporated to proteins. Arginine kinase activity was detected in soluble extracts from *T. cruzi* epimastigotes. This enzyme activity required ATP and Mg^{2+} and it was reverted by the addition of ADP.

BC-23
INHIBITION STUDIES OF CYSTEINE PROTEASES FROM *TRYPANOSOMA CRUZI* BY MOLECULAR MODELING

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Cysteine proteases from *Trypanosoma cruzi* have been validated as potential targets for anti-parasitic drugs. Cruzipain (cruzain), the major cysteine protease from *T. cruzi*, is a member of a polymorphic multigene family. A new isoform of cruzipain (cruzipain 2), displays marked differences in inhibitor affinity and substrate specificity when compared to cruzain, the family prototype (see accompanying abstract Reis et. al.). We have used Molecular Modeling methods to investigate interactions of inhibitors with the catalytic site of cruzain and cruzipain 2. The present work studies a new approach to estimate relative interaction affinities of enzyme inhibition, making use of known information from the crystal structures of complexes as well as from docked complexes. We then submit these complexes to an energy minimization. These minimized structures form the basis for calculating interaction variables that could be useful in deriving a quantitative model for predicting the interactions' affinity, using molecular mechanics calculations (THOR program). Using this methodology, we intended to correlate the molecular mechanics results with results of enzyme kinetics. The potent irreversible inhibitor of papain-like cysteine proteases, E-64 was chosen for this study. Spectrofluorimetric analysis were used to determine the inactivation constants of cruzain and cruzipain 2 with E-64. These experimental results show that E-64 is 30 times more potent for cruzain in comparison with cruzipain 2.We are now calculating the minimization energy of the complexes E-64 Cruzain , E-64 – Cruzipain2 and of the free enzymes by method Steepest Descent.

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BC-24
IMMUNOCHEMICAL CHARACTERIZATION OF CRUZIPAIN ISOFORMS DURING *TRYPANOSOMA CRUZI* DEVELOPMENT

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The major cysteine protease, cruzipain, is a member of a polymorphic multi-gene family, organized in tandem arrays in the parasite's genome. We had described the expression of cruzipain isoforms which diverge substantially (primary sequence) from the family's prototype cruzain, a cathepsin L-like enzyme. In an accompanying abstract (Reis et al.) we show that cruzipain 2 and cruzain indeed display different enzymatic specificities. In addition, differences in putative sites of N-glycosilation were also predicted, based on their gene sequences. In order to investigate the sub-cellular distribution and stage-specific expression of cruzipain isoforms, we raised policlonal antibodies against recombinant cruzipain 2. A fragment of cruzipain 2 extending from part of the Pro region to the C-terminal domain was obtained by PCR amplification of the full length gene. The purified fragment was cloned into the commercial vector p-GEX-4Ti and introduced in the *Escherichia coli* strain BL21DE3 for the expression of a fusion protein with glutathione-S-transferase. Soluble recombinant protein was purified by affinity column and analyzed by SDS-PAGE and Western blot using anti-GST serum. Most of the recombinant protein was present under the form of inclusion bodies, which were purified using previously described methods. Inclusion bodies with high contents of fusion protein were used to immunize rabbits. After two boosters, their serum was analyzed for the presence of anti-cruzipain 2 antibodies by a capture-immunoassay using cystatin C in the solid phase. Anti-serum 222 showed reactivity against recombinant cruzipain 2 and low cross reactivity against the major cruzipain (cruzain). Western blot analysis of extracts from the three developmental stages of the parasite showed strong reactivity in trypomastigotes and amastigotes (bands of Mr ~ 60 and 40 kDa) and no significant reaction was observed with

epimastigotes. The lower molecular band may indicate a protease that lost its C-terminal extension. Further analyses of the stage-specific expression of cruzipain 2 is under investigation. N-terminal sequence analysis should reveal if these antigenic molecules correspond to cruzipain 2, or alternatively, represent polymorphic variants from this subset of cysteine proteases.

Supported by CNPq, PADCT, Faperj.

BC-25
IDENTIFICATION OF PLASMENYLETHANOLAMINE AS A MAJOR COMPONENT OF THE PHOSPHOLIPIDS OF STRAIN DM 28C OF *TRYPANOSOMA CRUZI*

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Chaga's disease, caused by the trypanosomatid flagellate *Trypanosoma cruzi*, was first described by Carlos Chagas in 1909. This disease is endemic throughout Central and South America, very complex, with several different clinical manifestations that range from a nearly complete lack symptoms to severe and often lethal cardiomyopathy, megacolon or megaesophagus. The etiology of the clinical variability of Chaga's disease is not understood, but one of possible determining factors is the infecting strain of *T. cruzi*.

Plasmalogens are a unique class of glycerophospholipids with an alk-1-enyl ether substituent on the *sn*-1 position of glycerol, which are important components of mammalian cell membranes, and are specially abundant in peripheral nerves, brain and heart muscle.

In this study a novel phospholipid has been purified from strain Dm 28c of *T .cruzi* and characterized by FAB MS and NMR as a plasmenylethanolamine with a hexadec-1-enyl group in the *sn*-1 position and an approximately equimolar mixture of octadecenoate and octadecadienoate esterified to the *sn*-2 hydroxyl.

The TLC-separated plasmenylethanolamine reacted positively when probed with sera from patients with chronic Chagas' disease. Since plasmenylethanolamine of similar structure are abundant in mammalian cardiac and neuronal tissues, cross reactions between these epitopes may be a factor in the autoimmune pathology in the chronic phase of Chagas' disease.

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BC-26
IDENTIFICATION AND CARACTERIZATION OF USNRNPS *TRYPANOSOMA CRUZI*

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Trypanosoma cruzi is the etiologic agent of Chagas' Disease, which infection affects about 16-18 million individuals on endemic areas of Latin America. Although on the majority of vertebrates pre-mRNAs are processed by cis splicing, on Trypanosomes and Nematodes this process occurs by a different way called trans-splicing, on which a short transcript (about 40 nucleotides) called splice-leader (SL) RNA is transferred to the 5' acceptor pre-mRNA end. On the other hand, protein complexes involved on trans splicing reaction are formed by ribonucleoproteins and small RNA molecules. Theses small and abundant ribonucleoproteins are highly conserved and frequently target of autoantibodies present on sera from individuals with Autoimunes Diseases. All snRNPs involved in the formation of mRNA are classified on Sm group, it means that they have a small polypeptides region (11-29 KD) that is recognized by anti-Sm autoantibodies. Considering this information, we made use of purified rabbit anti-Sm antibodies to screen two cDNA libraries: one from epimastigote and another from amastigote form of *T. cruzi*, both in lambda UNIZAP phage. We have isolated two clones by in vivo excision from lambda Zap and after EcoRI/XhoI digestion the inserts showed app. 1000 and 900 bp, respectively, eventhough the recombinant proteins showed higher molecular weights than expected, also revealed by Western blot. Sequence analysis is in progress to check these results.

BC-27
HAEMOLYMPH AGGLUTINATION ACTIVITY OF FIVE TRIATOMINE'S SPECIES AGAINST RABBIT ERYTHROCYTES AND THREE STRAINS OF *TRYPANOSOMA CRUZI*

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Recently haemolymph lectin activity was demonstrated in *Rhodnius prolixus* and this galactose-binding protein was partially purified, in one step, by affinity chromatography. In this present study, the haemolymph lectin activity of five triatomine species: *Triatoma infestans*, *Panstrongylus megistus*, *Rhodnius neglectus*, *Rhodnius brethesi* and *Dipetalogaster maximus* has been investigated The haemolymph supernatant of all species showed, with different titers, an erythrocyte agglutinin activity, that was inhibited by several galactose-related sugars. The haemolymph agglutinin activity was also tested against three strains/clones of *Trypanosoma cruzi*: AM4167 (isolated from *Rhodnius brethesi* collected in the Amazon), Y (isolated from a Chagas' human case) and clone Dm28c (isolated from the marsupial, *Didelphis marsupialis*). The insects haemolymph showed different titers and patterns of agglutination against the culture forms of the *T. cruzi* strains. The haemolymph supernatant of *P. megistus* and *R. brethesi* agglutinated *T. cruzi* forming a network, immobilizing and killing the parasites However, the haemolymph supernatant of *T. infestans*, *D. maximus* and *R. neglectus* agglutinated the parasites forming small clumps,which in some cases were difficult to quantify. Preliminary results showed that the haemolymph proteins from the five strains of *T. cruzi* had different migration profiles in the electrophoresis-gels (SDS-PAGE).

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BC-28
GLYCOINOSITOLPHOSPHOLIPIDS (GIPLS) OF *TRYPANOSOMA CRUZI* COLOMBIANA STRAIN

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Glycoinositolphospholipids that are not linked to either protein or polysaccharide are major cell surface glycolipids in all trypanosomatids investigated to date. In previous studies we have characterized the molecular structure of GIPLs of different strains of *Trypanosoma cruzi*. Although the GIPLs share the same Man_4-GlcN-InsPO_4, core, they can be classified into two series on the basis of the substituent (ethanolamine phosphate or b-galactofuranose) on the third mannose residue (Man 3) distal to inositol (Carreira et al. 1996 *Glycoconjugate J 13*: 955-966). Now, we extended our investigation to the PI-oligosaccharide isolated from the major GIPL found in *T. cruzi* Colombiana strain. Structural analysis using chemical and FAB mass spectrometry methods showed that the major GIPL from Colombiana strain is formally related to the tetramannose type of GIPL found in Tulahuen strain, differing by the presence of 2-aminoethylphosphonate on the Man 3 instead of ethanolaminephosphate. The primary oligosaccharide sequence and the lipid composition of that Colombiana GIPL are, actually, under investigation.

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BC-29
GALACTOSE BINDING PATTERN ON *TRYPANOSOMA CRUZI* INFECTED AND NON INFECTED MOUSE HEPATOCYTE PLASMA MEMBRANE

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We have previously described that *Trypanosoma cruzi* invade and develop its life cycle within mouse hepatocytes culture (Porrozzi et al. 1997 *Mem Inst Oswaldo Cruz 92*: 117-120). Here we investigate whether macromolecules on the cell membrane, especially carbohydrate-containing compounds, that are responsible for a number of specific functions ocurring at cell surface, could be involved in recognition events of the *T. cruzi*-hepatocyte interaction. The presence of the galactose specific receptor has already been described in rat liver cells (Kempka & Kolb-Bachofen 1985 *Biochem et Biophy Acta 1008*: 114).

Hepatocytes obtained by collagenase digestion of mouse embryos livers were partially purified by centrifugation and cultivated as long term cultures in a defined medium. To investigate the surface distribution of galactose residues we incubated normal hepatocyte cells with RCA 120 - FITC or coupled to 15nm colloidal gold particles, for fluorescence and scanning electron microscopy (SEM), respectively. For transmission electron microscopy (TEM) uninfected *and T. cruzi* infected cells were (a) incubated with RCA 120 -colloidal gold conjugate at 4°C before glutaraldehyde (GA) fixation or (b) incubated with galactose-colloidal gold conjugate after fixation for 1 hr at 4°C.

Hepatocytes cultures preserved their original poligonal shape and bile canaliculi as characteristic of well differentiated liver parenchymal cells. Fluorescence patterns showed uniform distribution in the cell surface. SEM displayed colloidal gold particles uniformly distributed on the hepatocyte membrane. TEM also revealed a regular distribution of galactose residues in infected and uninfected fixed cell's surface. In live cells, colloidal gold particles were visualized in pits and invaginations of theirs surface but with a less intensity. When we observed *T. cruzi*

infected cells under both treatments, rare gold particles were observed over the hepatocyte plasma membrane. These differences in the expression of galactose residues between normal and infected cells seems to be directly related to the presence of the parasite. Additional protocols are being done in order to understand the decrease in the galactose binding sites in *T. cruzi* infected hepatocyte cells.

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BC-30
FUNCTIONAL EXPRESSION AND PURIFICATION OF *TRYPANOSOMA CRUZI* PHOSPHO-ENOL-PYRUVATE CARBOXYKINASE

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Phospho-enol-pyruvate carboxykinase (PEPCK ATP-oxaloacetate carboxylase transphosphorylating E.C.4.1.1.49) is a key enzyme in the intermediary metabolism of *Trypanosoma cruzi*. Sequence analysis of the parasite's PEPCK revealed differences compared to the homologous human enzyme. These differences were observed in the putative substrate binding sites, hence turning this enzyme into an interesting model for basic biochemical studies aiming to the development of specific anti -*T.cruzi* drugs.

Here we describe the cloning, functional expression and purification of recombinant PEPCK in *Escherichia coli*. The entire coding region of PEPCK was cloned into the expression vector pANEX, which consists of the vector pGExB without the gene encoding glutathione-S-transferase. The vector was used to transform *E. coli E1786* which is *pepck*$^-$ and malic enzyme deficient. After induction of the *tac* promoter with IPTG at 37^{o}C the expressed PEPCK enzyme was mainly insoluble. The inclusion bodies were purified and used for anti serum preparation. However, part of the recombinant enzyme was soluble and complemented the PEPCK deficient strain *E1786* when grown on minimum plates containing maltose. The purification of the soluble PEPCK was done by passing the cleared lysate over a hydroxyapatite column and the active fractions were then applied on a DEAE cellulose column where the pure and active enzyme was collected in the flow through. The specific activity of the pure recombinant enzyme was much higher than the enzyme purified directly from *T. cruzi*. This is very likely due to the fewer purification steps and reduced levels of proteases in *E. coli* when compared to the parasite. However, the km *versus* the substrate phospho-enol-pyruvate was similar irrespective to the origin of the enzyme. We are presently attempting to obtain crystals of the recombinant PEPCK.

This work received financial support from Pronex, CNPq, PADCT and Papes-Fiocruz.

BC-31
EXPRESSION OF A MARKER FOR INTRACELLULAR *TRYPANOSOMA CRUZI* AMASTIGOTES IN EXTRACELLULAR SPHEROMASTIGOTES

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We generated *Trypanosoma cruzi* cells expressing GFP by constructing a DNA cassette containing four elements: (i) a segment bearing the *T. cruzi* rRNA promoter, (ii) a segment containing the signals for addition of the SL to amastin RNA, (iii) the GFP gene and (iv) a fragment bearing the amastin 3' UTR plus a portion of the downstream region. The amastin 3' UTR plus IR was inserted immediately downstream from the GFP gene in anticipation that this sequence would result in a higher level of GFP expression in amastigotes than in epimastigotes and trypomastigotes. The GFP cassette was inserted into a plasmid containing the TCR27 and Neo^r genes and electroporated into *T. cruzi* Tulahuén epimastigotes. Four clones were selected from agar plates containing G418.

Confocal micrographs of different life-cycle stages of *T. cruzi* containing the GFP cassette integrated into a TCR27 allele shows GFP-expressing amastigotes inside cultured human renal adenocarcinoma cells where bright green fluorescence is readily apparent. All of the amastigotes have approximately the same high level of fluorescence. We also observed GFP-expressing parasites in axenic culture. The flagellated epimastigotes display a uniformly weak fluorescence, consistent with our earlier observation that the presence of the amastin 3' UTR + IR downstream of a luciferase gene greatly diminishes luciferase expression in epimastigotes. About 1-3% of the parasites in these axenic cultures, that are morphologically indistinguishable from intracellular amastigotes, express GFP at the same high level as in intracellular amastigotes. Thus, a strong evidence that extracellular spheromastigotes are equivalent to intracellular amastigotes at the gene expression level is presented.

Another possible application for the GFP-expressing parasites is to use them to study the efficacy of experimental anti-*T. cruzi* drugs in laboratory animals, because of the relatively easy visual detection of the fluorescing organisms. However, after several attempts of infecting Balb/c mice with trypomastigotes expressing GFP we were

unable to detect parasites either under light or fluorescence microscopy. Our inability to induce detectable infections in heart tissue likely was the result of this previously virulent Tulahuén strain having become attenuated during more than a decade of maintenance in cell culture and mice.

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BC-32
EXPERIMENTAL INFECTION OF OLD WORLD MONKEYS WITH *TRYPANOSOMA CRUZI* LINEAGE 2: RE-EVALUATION AFTER 14-17 YEARS

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Intriguing aspects of *Trypanosoma cruzi* biology are the population heterogeneity of the parasite. Molecular and biochemical data generated by several different techniques showed that the *T. cruzi* taxon is composed of heterogeneous sub-populations. No correlation has been established between the distinct genotypes or phenotypes and the different aspects of *T. cruzi* and American trypanosomiasis. Recently, the characterization of two different loci, the mini-exon and the rRNA gene, of several *T. cruzi* isolates has clustered the stocks into two genotypic lineages. Molecular epidemiological studies performed in four distinct geographical areas in Brazil, analyzing 86 field recently-isolated stocks from humans and triatomines, showed a preferential association of lineage 1 with the domestic cycle and therefore, with the human host. Furthermore, after analyzing the sylvatic cycle that is present in the Poço das Antas Biological Reserve we reached the conclusion that the *T. cruzi* isolates that have been infecting monkeys belong to the same lineage 1. To investigate whether this phenomenon is a reflect of a specific action of the primates as biological filters to lineage 1, our group decided to perform the following experiments: 1. experimental infections in New World monkeys with the two distinct lineages and 2. analysis of 7 Old World monkeys that have been infected with a lineage 2 strain of *T. cruzi* (Colombian strain) for 14 to 17 years. The results concerning the latter proposal are presented herein. Seven male *Macaca mulatta* were infected subcutaneously with approximately 1500 *T. cruzi* metacyclic trypomastigotes/kg weight (Colombian strain) in 1981 (3) and 1984 (4). The patent parasitemia, detected by direct microscopy, could be observed until 2 months after infection. Recently, blood from these monkeys were submitted to hemoculutre and were all negative. PCR analysis, trying to amplify the variable region of the minicircle molecule, revealed that only two (28% - 2/7) were positive for *T. cruzi*. Although the clearance of *T. cruzi* should not be expected in infected monkeys, it seems that 5 (72% - 5/7) were able to eliminate the parasite when the fact was analyzed by either hemoculture and PCR. Future experiments will determine the reasons why most of the monkeys managed to eliminate *T. cruzi* lineage 2.

BC-33
EFFECTS OF SURAMIN ON THE ECTO-ACTVITIES IN *TRYPANOSOMA CRUZI*

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Trypanosoma cruzi, the etiologic agent of Chagas' disease can subvert host cell signal transduction pathways for their own benefit. In some cases, one of the goals of the subversion strategy is to gain access to the host cells. Current evidence indicates that *T. cruzi* penetrates culture mammalian cells by a unique mechanism distinct from phagocytosis and this invasion process occurs in an energy-dependent manner. In this study we investigated the effects of suramin on the ecto-activities of live *T. cruzi* (colombiana strain). Suramin is a potent non-selective P_2 antagonist receptor. It is a symmetrical polysulfonated naphthylamine derivative of urea widely used since the 1920s for prophylactic treatment of human trypanosomiasis in Africa (Voogd et al. 1993 *Pharmacol Rev 45*: 177-203). We observed that increasing concentrations of suramin inhibits progressively the ecto-ATPase activity of *T. cruzi*, however this reagent does not inhibit the ADPase, 5'-nucleotidase and phosphatase activitites. We also observed here that ATP minimizes the inhibitory effect promoted by suramin. When intact cells are incubated in a medium containing 1 mM of ATP in the presence of increasing concentrations of suramin, the concentration of suramin necessary to inhibit 50% of the ATPase activity was 0.8 mM. Now, when the cells are incubated in a medium containing 10 mM of ATP, Ki for suramin increased to 3.1 mM. It has been described (Chen et al. 1996 *Br J Pharmacol 119*: 1628-1634) that suramin has dual properties of acting as ecto-ATPase inhibitor and of causing receptor blockage. The decrease of the inhibition, by ATP, of the ecto-ATPase activity in *T. cruzi*, might suggest that ATP and suramin should compete by the same receptor; however, it remains to be elucidated.

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BC-34
ECTO-KINASE AND SECRETED KINASE ACTIVITIES IN *TRYPANOSOMA CRUZI, LEISHAMANIA TROPICA* AND LOWER TRYPANOSOMATIDS

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During its life cycle trypanosomatids face several different environmental conditions in invertebrate vectors, vertebrates and plants. In the case of human parasites, trypanosomatids should be able to escape host mechanisms of defense. Several molecular mechanisms have been described in the last few years which ensure parasite infection despite of host efforts. Protein phosphorylation-dephosphorylation is one of the most powerful mechanisms able to control cell activities. A series of studies have demonstrated the role protein phosphorylation may play in the coordination of parasite defenses. A growing body of evidences demonstrate that several enzyme activities located on the surface of these parasites play an essential role both in parasite's survival and in its ability to invade host cells. Ectophosphatases are enzymes able to dephosphorylate free phosphoaminoacids as well as those located inside phosphoproteins. These enzymes, together with ecto-ATPases, received a growing attention in the last few years. In this study we tested intact cells of *Trypanosoma cruzi* (Y strain), *Leishmania tropica*, *Herpetomonas muscarum muscarum* and *Phytomonas serpens* and we showed the presence of an ecto-kinase activity on the surface of all these trypanosomatids, as well as a secreted protein kinase activity, which are measured from parasites incubated with dephosphorylated casein. The pre-incubation of parasites with casein did not affect the ecto-kinase activity although it increased by 2 fold the kinase activity found in parasites' supernatants. From the parasites tested, *P. serpens* exhibited the greatest ecto-kinase activity (85.3 pmol Pi / mg . min) and also the highest casein stimulated kinase secretion (541.0 pmol Pi / mg . min). The other ecto-kinase and secreted kinase activities were 34.3 and 455.9 pmol Pi / mg . min, respectively for *H. m. muscarum*, 25.2 and 341.7 pmol Pi / mg . min, respectively for *T. cruzi* and 65.8 and 284.4 pmol Pi / mg. min, respectively, for *L. tropica*.

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BC-35
DOWNREGULATION OF GENE EXPRESSION BY THE REPETITVE ELEMENT SIRE IN *TRYPANOSOMA CRUZI*: THE CASE OF TCP2B RIBOSOMAL PROTEIN

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It is generally accepted that trypanosomes regulate gene expression mainly at the post-transcriptional level. There are four TcP2b loci in the genome of *T.cruzi* (H6.4, H1.8, H1.5, H1.3). One of these, H1.8, contains two identical genes in tandem separated by a repetitive element SIRE positioned in front of each gene. SIRE is absent in the other loci. SIRE inserts its self between the Py region and the AG acceptor site, disrupting the typical trans-splicing signal of this P2b gene. Nevertheless, the H1.8 gene transcript is trans-spliced by the use of a signal present in the 3'end of SIRE. Comparison of this signal with the typical P2b signal, showed that the Py sequence in SIRE is much shorter than the Py regions present 5' to the other TcP2b genes. Moreover, cDNA cloning and RT-PCR analysis indicated that H1.8 gene is the less abundant mRNA in the TcP2b mRNA population. This finding suggested that SIRE may downregulate the H1.8 gene expresssion at the level of RNA maturation. We analized this possibility using constructs with CAT as reporter gene. We constructed two reporter plasmids, one containing the typical 5' region of TcP2b genes (HX1 region) and another with the same region plus the inserted SIRE (HX2 region), both plasmids used the SV40 region for 3'end processing because, being a non trypanosome sequence, it is efficiently processed by trypanosomes. Transient transfection experiments indicated that plasmids containing HX2 were 45% less efficient in trans-splicing than plasmids containing HX1, indicating that SIRE signals may be, indeed, implicated in downregulation of H1.8 gene. We conclusively demostrate that the repetitive element SIRE includes a functional mRNA processing signal, albeit of low efficiency that downregulates the expression of the H1.8 gene. This may be a general property of SIRE because this element is associatted to other well characterized *T.cruzi* genes in a similar manner.

BC-36
DIFFERENTIAL IMMUNOSCREENING OF *T. CRUZI* AMASTIGOTE ANTIGENS

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The majority of persons chronically infected with *Trypanosoma cruzi* are asymptomatic and remain in the so called indeterminate phase of the disease. In 20-30% of infected individuals however, cardiac and gastrointestinal manifestations typical of chronic Chagas disease occur. As a long term goal for the present study, we propose to evaluate a large number of recombinant antigens derived from *T. cruzi* amastigotes, according to the humoral and cellular immune responses they elicit in two groups of chronically infected chagasic patients: asymptomatic persons and patients with chagasic cardiopathy. Using an amastigote cDNA library and pools of sera from these two groups of patients, we have isolated 30 positive clones. The vast majority of them react equally with antibodies from both pools. In this first screening however, where 50,000 p.f.u. were analysed, two clones seem to react more strongly with pool of sera from patients with cardiophathy and one shows a more strong reaction with sera from asymptomatic persons. The cDNA inserts in various positive clones have been characterized by restriction mapping and partial nucleotide sequences of eight cDNAs have been determined so far. As expected, some clones were found to encode ribosomal proteins and heat shock proteins, but sequence comparison of four cDNA clones showed no homology with previously known sequences. These recombinant antigen are now in the process of being tested by Western blotting to provide a better estimation of the levels of reactivity with serum from each patient. We hypothesize that two classes of antigens could be found: (*i*) "protective" antigens that stimulate B and or T- cell responses controlling the parasitic infection, in asymptomatic patients; (*ii*) "cross-reactive" antigens containing epitopes shared by the parasite and mammalian cells that will be more likely identified using sera from cardiac patients which may present autoimmune reactions.

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BC-37
DIFFERENTIAL EXPRESSION OF SERUM AMILOYD A3 GENE BY MURINE INFLAMMATORY MACROPHAGES EXPOSED TO GLYCOSYLPHOSPHATIDYL INOSITOL-ANCHORED MUCIN-LIKE GLYCOPROTEIN PURIFIED FROM *TRYPANOSOMA CRUZI* TRYPOMASTIGOTES

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Comparative analysis of cDNA fragments from mRNAs isolated from macrophages cultured in the presence of medium alone, glycosylphosphatidylinositol anchore mucin-like glycoproteins isolated from *Trypanosoma cruzi* trypomastigotes (tGPI-mucins) and/or IFNg was performed by using the Differential Display technique. Most fragments were identical in all four cDNA preparations fractionated in a sequencing gel. Nevertheless, we identified several fragments that were present on cDNA prepared from activated macrophages that were absent in the cDNA preparation from resting macrophages. The differential cDNA were extracted from sequencing gel, cloned, sequenced and used to hybridize to mRNA extracted from macrophage populations exposed to different stimuli. Analysis on a genebank revealed that among the different cDNA fragments one presented high homology with the serum amyloid A3 (SAA3) protein. Our Northern blot assays showed augmentation of SAA3 mRNA expression by inflammatory macrophages exposed to live trypomastigotes or parasite glicolipids. In addition, we found that enhancement of SAA3 mRNA expression was dependent on protein synthesis by macrophages exposed to tGPI-mucins. Finally, we studied whether *T. cruzi* induces expression of SAA3 mRNA in vivo. Our results showed that liver, spleen and hearts, from animals acutely infected with *T. cruzi*, express high levels of SAA3 mRNA. More importantly, the expression with SAA3 in the different organs was closely associated with the recruitment of inflammatory infiltrates. These findings indicate the possible involvement of SAA3 protein on immunopathology of Chagas' disease and establish a new infectious disease experimental model to study the physiological role of this acute phase protein.

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BC-38
DETECTION, PURIFICATION AND MASS SPECTROMETRIC IDENTIFICATION OF THE APYRASE OF *TRIATOMA INFESTANS*

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The insect-vectors of human diseases have developed the hematophagy in a process of evolution associated with the presence of various pharmacologically active substances in their salivary glands. The insect feeding hability, here understood as its capacity to overcome the host homeostatic response, can be related to various compounds secreted in the saliva (Ribeiro, 1995). The apyrase, a diphosphohydrolase that removes Pi (inorganic phosphate)

from ATP and ADP, shows a potent platelet anti-aggregation function. Its presence in the saliva has been related to the fluidity of the blood essential for the insect feeding and thriving.

In this study, we report the presence of apyrase in the saliva of *Triatoma infestans*, the main vector of *Trypanosoma cruzi* to the human host in Brazil and other countries of South America. The apyrase activity has been detected in the insect saliva by the Pi quantification method of Fiske & Subbarow. This activity was partially purified by Oligo$(dT)_{12-18}$-Cellulose^{â} chromatography. The eluted proteins were analysed by SDS-PAGE electrophoresis. The main silver stained protein bands were submitted to identification by means of the peptide mass fingerprinting technique. Shortly, the protein bands were excised from the gel and digested with trypsin, followed by elution of the fragments, desalting through Poros resin and determination of peptide masses by using a Perkin Elmer-Sicex API300 electrospray-triple quadrupole mass spectrometer. The experimentally determined masses were searched (Peptide Search, EMBL, Heidelberg, Germany) against theoretical masses of peptides produced by *in silico* digestion of proteins found in databanks. This approach allowed the identification of a 88 kDA protein pieces of gels were treated with trypsin and the protein fragments were analysed by the method of finger printing peptide mass using the electron spray method. This approach allowed identification of a protein in the upper band, with approximately 62 kDa, which showed homology with the *Aedes aegypti* (Champagne et al., 1995) counterpart. The kinetics of the enzyme activity showed features similar to those reported for other apyrases, except that the *T. infestans* apyrase was shown to be Mg^{++} dependent. The gene encoding for the apyrase of this insect-vector of Chagas' disease is under study for sequencing.

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BC-39
DETECTION OF *TRYPANOSOMA CRUZI* IN BLOOD SPECIMENS OF PATIENTS WITH CARDIOMYOPATHY AND CHRONIC CHAGAS' DISEASE

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Detection of *Trypanosoma cruzi* in blood is important to evaluate the status of patients with cardiomyopathy and chronic Chagas' disease. Direct parasite detection is difficult in the intermediate or chronic phase of the disease because of their low concentration in blood. On the other hand, serologic tests for diagnosis of chronic Chagas' disease have high sensitivity but lack specificity because of antigenic cross-reactivity with other parasites. In addition,, these methods only detect antibodies against the parasite and not the presence of the parasite itself. There is actually a considerable amount of data about the utility of the polymerase chain reaction (PCR) in detection of *Trypanosoma cruzi* in blood specimens by amplification of sequences from nuclear DNA, kinetoplast DNA, nuclear intergenic ribosomal DNA, and ribosomal RNA. In a previous work, we amplified a genomic DNA sequence of *Trypanosoma cruzi* in blood samples from patients with chronic Chagas' disease. In the present study we correlate data from serologic tests, haemoculture, and PCR in blood from patients, in the chronic phase of the disease, with miocardyopathy clinically diagnosed. Blood samples from 54 patients were analyzed by haemoculture (LIT medium); PCR (DMID, 30 (3): 183-186, 1998); (indirect immunofluorescence (IIF), (positive >1:32 dilution) (Biocientífica); hemmaglutination (positive >1:28 dilution) (Biochagas, Biocientífica); and enzyme like immunosorbent assay (Abbot). Patients were considered serology-positive when the serum reacted in at least two of three tests. Our results show: a) 21% of patients serology and parasitology negatives; b) 19% serology and parasitology positives; c) 56% serology positives and parasitology negatives, that would be attributable to persistence of the immune response; and d) 4% serology negatives and PCR positives, that could be recently reinfected patients. We can conclude that PCR method may be useful as prognostic factor in order to evaluate the disease evolution in patients with cardiomyopathy in the chronic phase.

BC-40
CONCORDANCE OF MICROSATELLITE ANALYSIS AND ISOZYME CLASSIFICATION IN *TRYPANOSOMA CRUZI*

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We have recently studied the genetic diversity of *Trypanosoma cruzi* using eight different (CA)n microsatellites (PNAS 95: 3776, 1998). In 30 different strains and clones we found a remarkable degree of genetic polymorphism. Moreover, our microsatellite data supported the hypotheses that *T. cruzi* is diploid and that it undergoes predominant clonal evolution. Several strains, especially those isolated from non-human sources, showed more than two alleles

in many loci, demonstrating that they were multiclonal. To further evaluate the usefulness of microsatellite typing of *T. cruzi* we compared it with the more traditional and widely used isoenzyme classification, together with multiprimer RAPD fingerprinting. For that, we used a sample of 21 strains that had been typed in Montpellier with a battery of 22 isozyme loci and a set of 50 RAPD primers. These strains were considered representative of the whole phylogenetic diversity of *T. cruzi* and included representatives of all the three principal zymodemes of Miles, as well as of the major clones identified by Tibayrenc and Ayala (1988 *Evolution 42*: 277). The 21 strains were then analyzed in Belo Horizonte with 8 microsatellite loci, without knowledge of the isozyme and RAPD classification. No single multilocus genotype was seen more than once, confirming the high degree of genetic diversity of the parasite. Four strains that showed loci with more than two alleles were considered multiclonal and were dropped from further analysis. To make phylogenetic inferences about the remaining 17 apparently clonal strains, we assumed a stepwise mutation model for the microsatellites and used as a measure of genetic distance between any two strains the minimum number of mutational steps necessary to transform one into the other. We then used maximal parsimony to build an unrooted Wagner network. When the isozyme data were mapped onto the network, we observed absolute concordance of the two sets of data. All strains belonging to the same zymodeme clustered together in branches of the tree. This perfect agreement between microsatellite and isoenzyme/RAPD data confirms the high levels of linkage disequilibrium observed in the *T. cruzi* genome as a consequence of predominant clonal evolution. This does not rule out the possibility of occasional events of hybridization, already recorded by several authors. Furthermore, it validates microsatellite analysis as a technique of extraordinary usefulness for genetic studies of *T. cruzi*.

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BC-41
CLONING OF TWO MEMBRANE PROTEINS OF *TRYPANOSOMA CRUZI* GLYCOSOME: USE AS A ANTIGEN FOR CHAGAS DISEASE TEST

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The present work describes the cloning of the genes of two glycosome membrane proteins of *Trypanosoma cruzi* (cepa EP) by immunologic screning of a epimastigotes cDNA library. Antibodies were obteined by absorptin of sera of chronic chagasic patients to low molecular weight (7-40 kDa) glycosome were brane proteins. Antibodies specific of glycosome membrane proteins were eluided by changing pH and used in the screenig.The protein with molecular weights of 7-40 kDa were the most specific and they did not present cross-reactivity with sera related parasitary diseases (Concepcion 1996 *Mem Inst Oswaldo Cruz 91*: 256). Seven different clones were obtained. Two clones were sequenced, WDG-5 and CDG-4. WDG-5 poseed the SKL sequence and a open reading frame of 71 aminoacids residues containing a Zinc binding motive with 4 Cys arranged in pairs. This motive has been described for ribosomal proteins of S29 type, chaperones and some integral proteins involved in polypeptides translocation. CDG-4 coded for a 200 a.a. proteins wich did not present significative homology with any known proteins. It only presented a cluster of positive changes which has been reported for import into the glycosome.

We evaluated a diagnostic method of Chagas disease using two recombinant glycosomal membrane proteins of *Trypanosoma cruzi* (EP strain) as antigen with 30 sera of chagasic patients in chronic phase from different endemic areas of Venezuela. Sera samples were analyzed by Western blotting (WB) and ELISA. As a control we used extracts of the epimastigote forms of the parasite. In all the tests realized, we also used sera from patients without any known infection as well as from patients suffering from other parasitic and microbial diseases such as (20) leishmaniasis, (6) amebiasis, (5) tuberculosis, (5) HIV, (12) toxoplasmosis, (9) rubeola, (6) cisticercosis, (6) lupus eritematoso, (4) syphilis and (11) hepatitis.

There was no evidence of croos-reactivity with a polyclonal obtained from glycosomal membrane recombinant proteins when extracts from *Leishmania mexicana* were used as antigens. In conclusion, the recombinant protein WDG-5 y CDG-5 are potentially useful antigens in the serological diagnosis of Chagas disease due to high specificity and sensitivity and the lack of cross-reactivity with other closely related parasitary diseases.

BC-42
CLONING BY THE TWO HYBRID SYSTEM OF A NOVEL CYCLIN (TCCYC2) THAT ASSOCIATES TO THE TZCRK1 PROTEIN OF *TRYPANOSOMA CRUZI*

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In eukaryotic organisms G1/S and G2/M cell cycle transitions are controlled by the activity of cyclin-dependent protein kinases (CDKs). A cdc2-related protein kinase, TzCRK1, was cloned from the protozoan parasite *Trypano-*

soma cruzi. TzCRK1 encodes a 33 kDa protein sharing a high degree of identity (> 78 %) with *T. brucei* CRK1, *Leishmania mexicana* CRK1 and *T. congolense* CRK1. The two hybrid technique was used to identify and clone proteins that associate to this kinase.The tzcrk1 gene was subcloned in the pBTM116 vector in fusion with the lex A DNA-binding domain. This construction was transformed in the L40 yeast strain which has two independent reporter genes, lacZ and HIS3, under the control of the minimal GAL1 promoter fused to multimerized lexA binding sites. This yeast strain was then transformed with a *T. cruzi* epimastigote cDNA library constructed in the pVP16 yeast expression vector. Aproximately twenty different clones his+/lacZ+ were identified. All the clones were sequenced and analysed using the Blast program. One of the clones presented identity with the PREG protein from *Neurospora crassa* and the PHO80 protein from *Saccharomyces cerevisiae (Sc)*. These proteins belong to the cyclin family. The PHO85 CRK protein from *Sc.* is able to bind with different cyclins, PCL1, PCL2 and PHO80. The association with the former two cyclins appear to be involved in the regulation of G1 stage of the cell cycle, while the binding with the PHO80 protein form a complex that phosphorylates transcription factors which control the expression of enzymes related to phosphate metabolism. These results suggest that the TzCRK1 protein could be involved in cell cycle progression and/or in other processes such as phosphate metabolism.

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BC-43
CLONING AND SEQUENCING OF *TCCB*, A GENE ENCODING A *TRYPANOSOMA CRUZI* CATHEPSIN B-LIKE PROTEASE

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Trypanosoma cruzi contains high levels of cysteine protease activity, most of it associated with cruzipain, a cathepsin L-like protease. We have characterized another cysteine protease activity in cell-free extracts of epimastigote, amastigote and trypomastigote forms of the parasite. The protease that displays this activity has been purified and showed to be an acidic protease of 30 kDa with broad substrate specificity. Its N-terminal amino acid sequence is very similar to those proteases of the cathepsin B class (Garcia et al. 1998 *Mol Biochem Parasitol 91*: 263-272).

To clone the gene encoding this *T. cruzi* cysteine protease, a polyclonal antibody was raised against the purified enzyme and used to screen an epimastigote cDNA library. A 1.2 kb cDNA was obtained, cloned and sequenced. This clone was used to isolate a 4.6 kb fragment from a genomic library containg the complete ORF of the gene, named *T. cruzi* cathepsin B (*tccb*). The gene is present as a single copy in the genome of the parasite and it is expressed in all of life cycle stages as a transcript of 2.3 kb. The ORF of the *tccb* gene is 1002 bps long and predicts a protease of 37 kDa with 333 amino acids. The encoded protease has a short hydrophobic pre-region of 18 amino acids, followed by a pro-region of 71 amino acids. Its predicted translation product was found to be very similar to the mature domain of several cathepsin Bs, having 62% and 60% identity with the *Leishmania mexicana* and *Leishmania major* cathepsin B-like proteases, respectively, and 56% with the human counterpart. The same sequence is less identical with cathepsin L enzymes: the identity with the *Trypanosoma brucei* pTCP-F1 clone and cruzipain being 23% and 36%, respectively. The *T. cruzi* cathepsin B-like protease may play important roles in crucial metabolic and cellular events.

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BC-44
CLONING AND CHARACTERIZATION OF A *TRYPANOSOMA CRUZI* METACYCLIC TRYPOMASTIGOTE STAGE SPECIFIC GENE

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The differentiation of *Trypanosoma cruzi* epimastigotes into metacyclic tripomastigotes occurs in the intestinal tract of triatomines. Metacyclogenesis of *T. cruzi* can be performed under *in vitro* conditions using the chemically defined TAU 3AAG medium (Contreras et al. 1985 *Mol Biochem Parasitol 16*: 315). During the metacyclogenesis, genes are expressed at differents times and the study of these stage-specific genes is important for the understanding of the mechanisms involved in the regulation of gene expression. We have recently developed a method (Representation of Differential Expression - RDE) for the amplification and cloning of *T. cruzi* stage-specific genes (Krieger & Goldenberg 1998 *Parasitol Today 14*: 163). It is an adaptation of the representational difference analysis method - RDA (Lisitsyn et al. 1993 *Science 259*: 946) and it is based on the principles of subtractive hybridization followed

by PCR amplification. We have applied RDE for studying genes expressed by trypomastigotes obtained under *in vitro* conditions. The first step involves obtaining tester and driver cDNA populations for the subtractive amplification. Then, we have generated cDNA (RT) by reverse transcription of polysomal RNA extracted from trypomastigotes (tester) and epimastigotes growing in LIT medium (driver) of *T. cruzi* Dm28c, followed by PCR (RT-PCR). The population of tester molecules was hybridized to an excess of driver molecules and non-hybridized molecules were selectively amplified by PCR. This subtractive amplification procedure was done three times using an increasing excess of driver molecules. Later, the molecules obtained were cloned in plasmid Bluescript. Each clone was hybridized with epimastigote RNA probe to check its specificity. Northern blot analysis confirmed that clone #10 is expressed mainly by trypomastigotes. We are presently characterizing the gene corresponding to this clone in terms of its nucleotide sequence, expression and organization within *T. cruzi*.

Supported by Pronex, PADCT, CNPq, Capes and Papes-Fiocruz.

BC-45
CLONING AND CHARACTERIZATION OF 24H-AD: A GENE EARLY EXPRESSED DURING *TRYPANOSOMA CRUZI* METACYCLOGENESIS

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Previous work from our laboratory resulted in the development of chemically defined conditions that mimic the metacyclogenesis process of *Trypanosoma cruzi* (Contreras et al. 1985 *Mol Biochem Parasitol 16*: 315). Under *in vitro* conditions, differentiating epimastigotas adhere to the culture flasks and are released to the culture medium upon transformation into metacyclic trypomastigotes. We have recently developed a method for the amplification and cloning of *T. cruzi* genes specifically expressed at different times of the metacyclogenesis process (Krieger & Goldenberg 1998 *Parasitol Today 14*: 163-166). The method, named Representation of Differential Expression (RDE), is based on the PCR amplification of DNA sequences unique to a given cell population (tester), after hybridisation to an excess of DNA from a related cell population (driver) to remove sequences common to tester and driver populations. In order to characterise genes expressed at different times of the metacyclogenesis process, we have used cDNAs obtained from polysomal mRNAs of 24h-adhered epimastigotes and exponentially growing epimastigote as tester and driver populations, respectively, in a RDE procedure. A gene (24h-Ad) was fully sequenced and characterised in terms of its genomic organisation. Search on GenBank sequences showed that none of them displayed homology to this gene. Southern blot analysis and pulsed field gel electrophoresis indicated that it exists as low copy number gene in *T. cruzi* Dm28c. Restriction map of the genomic clone indicates that there are at least three copies of 24h-Ad gene located within tandem repeats of 2,8 Kbp in the genome of *T. cruzi*. We have observed that the 2,8 kbp repeats contain a unknown gene in addition to the 0,6 kbp 24h-Ad gene, leaving an intergenic regions of short length between these two genes. Northern blot analysis showed that neither the 630bp mRNA transcribed from the 24h-Ad gene nor the 1,3 Kb mRNA transcribed from the unknown gene are present in the polyribosomal fraction of epimastigotes and metacyclic trypomastigotes forms. We are presently sequencing the associated gene in order to search for homologous sequences in the GenBank and to determine its precise location within the genome of *T.cruzi.*

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BC-46
CLONING AND ANALYSIS OF A GENE ENCODING A BETA TYPE SUBUNIT OF *TRYPANOSOMA CRUZI* PROTEASOME

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The Proteasome is the major cellular proteinase complex of all eukaryotic cells which plays a significant role in a variety of physiological processes. The 20 S Proteasome is a dimer consisting of four stacked rings, each containing seven subunits. In eukaryotes several different proteasomal subunits ranging in molecular mass from 21 to 35 kDa are known, which can be divided into two classes, alpha-type or beta-type, based on their homology to the two different subunits, alpha and beta, of the 20 S proteasome of the archeon *Thermoplasma acidophilum*. In this work, the *T. cruzi* homologue of LMPX gene denominated *tcbeta1* were cloned. The gene encode a protein of 22kDa, which is 65% identical to the rat and human LMPX protein. Using this fragment as a probe, the cDNA clone have been isolated. Preliminary Northern blot analysis indicates the presence of a single 1.5 kb transcript in epimastigote forms. Southern analysis and hybridization of labeled *tcbeta1* genomic sequences to chromosome preparation reveals a single locus on the chromosome 20.

BC-47
CHARACTERIZATION OF THE *TCRAB*7 GENE PRODUCT AS A GTP-BINDING PROTEIN IN *TRYPANOSOMA CRUZI*

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The rab7 gene of *Trypanosoma cruzi* (*tcrab*7) has already been sequenced and characterized in our laboratory. It is present in the genome of this trypanosomatid as a single copy gene. After experiments of transfection we obtained a single transformant clone (B6) that is partially knocked out for rab7 gene and showed morphological alterations at the anterior region of the parasite. Besides, we obtained transformed cell lines stably super-expressing Rab7 protein (pTAG) and a similar protein but without the last three C-terminal residues (pDCXC). This Rab7 mutant fails to associate with membranes. Rab7 protein in *T. cruzi* CL Brener WT, pTAG and pDCXC is located at the anterior region of the epimastigote cells. Now we characterized TcRab7 protein by assessing its ability to bind the nucleotide GTP. First, we produced and purified the fusion protein TcRab7/GST in the bacteria *Escherichia coli* BL21. The Gluthatione-S-Transferase (GST) fusion protein was cleaved with thrombin and the GST part was removed by affinity chromatography on GSH Sepharose 4B. After cleavage, we determined by SDS-PAGE that TcRab7 is produced in this bacteria as a protein of, aproximately, 25 kDa. The ability of this protein in binding GTP was determined by thecnique of GTP-blotting. This approach consist of renaturation of the protein fixed in the nitrocelulose filter and incubation of it with radiolabeled GTP. As expected, TcRab7 produced in bacteria shows GTP-binding activity, similarly to Rab proteins of other organisms. So, we determined if the native protein of *T. cruzi* could bind GTP. We used a polyclonal antibody raised against TcRab7 protein to immunoprecipitate the native protein and the super-expressed protein present in total protein extracts of the *T. cruzi*, CL Brener clone (wild type, B6, pTEX, pTAG and pDCXC). We, first, determined by Western blot that the antibody recognized the fusion and the native protein and, by northern blot, that the messenger RNA of *tcrab*7 was being producing in the studied cells as a 1.7 kb product. The immunoprecipitation experiments developed with radiolabeled GTP demonstrated that all of the cell lines above mentioned have proteins of, aproximately, 25 kDa that can bind this nucleotide. This finding suggest that TcRab7p is functional as a G protein in *T. cruzi*.

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BC-48
CHARACTERIZATION OF THE MYO-INOSITOL TRANSPORT SYSTEM IN *TRYPANOSOMA CRUZI*

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Inositol, found in animals and plants, have been cited as essential in the growth of micro-organisms and animals. Its action rests in the formation of a complex set of inositol-containing lipids, including phosphatidylinositol and its phosphorylated derivatives involved in cell signaling or in the biosynthesis of glycosilphosphatidylinositol anchors and glycosylinositolphospholipids. We have used fluorinated-myo-inositol analogues (nFIns), to study the inositol metabolism in *Trypanosoma cruzi*. We found that some of the analogues had an inhibitory action on the phosphoinositides synthesis and cell proliferation. Those inhibitors did not entered the cells, suggesting that the action of nFIns were at membrane level probably blocking an inositol transport protein. This fact prompted us to characterize the inositol transport system in *T. cruzi*. Our results showed that inositol uptake is linear up to 30 min and rapidly transformed in inositol-phosphates and inositol-lipids. Other hexoses had no effect on inositol transport showing that this transporter is specific for myo-inositol. The transport is energy-dependent since inhibitors of the electron transport chain inhibited the inositol uptake suggesting that na electrochemical gradient is necessary for the inositol transport. It is also Na+-dependent, since it was partially inhibited by 5mM furosemide and 3mM ouabain. Furthermore, sodium free buffers, where Na+ was replaced by choline or by potassium showed a significant decrease in the inositol uptake. Our data suggest that inositol uptake by epimastigotes occurs in a symport with Na+. The observed energy requirement might be needed to maintain the Na+ gradient thus configuring a secondary type of active transport.

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BC-49
CHARACTERIZATION OF a-*N*-ACETYLGLUCOSAMINYL TRANSFERASE ACTIVITY IN DIFFERENT STRAINS OF *TRYPANOSOMA CRUZI*

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We have previously identified a novel series of *O*-*N*-acetylglucosaminyl-linked oligosaccharides from surface sialoglycoproteins of *Trypanosoma cruzi* G and Y-strains (Previato et al.1994. *Biochem J 301*: 151-159, 1995 *J Biol Chem 270*: 7241-7250). More recently, we have characterized in microsomal membrane preparations from epimastigote and trypomastigote forms of *T. cruzi* (Y-strain) the activity of a UDP-*N*-acetylglucosamine:polypeptide-a-*N*-acetylglucosaminyltransferase (Previato et al. 1998 *J Biol Chem 273*: 14982-14988). This glycosyltransferase is involved in the attachment of *N*-acetylglucosamine units (GlcNAc) to the peptide backbone, the first step of the biosynthesis of the *O*-glycan chains of *T. cruzi* sialoglycoproteins. In the present study, we have done a comparative investigation of the activity of this novel glycosyltransferase in a range of *T. cruzi* strains. We have demonstrated that the microsomal membrane preparations of Y, G, CL-Brener, Tulahuen and Dm28c strains incorporate similar amounts of *O*-*N*-[^{3}H]GlcNAc from UDP-[^{3} H]GlcNAc when the synthetic peptide $K_2P_4T_8$ was used as substrate. Interestingly, the incorporation of [^{3}H]GlcNAc into the $K_2P_4T_8$, using a microsome preparation from Colombiana strain as transferase source was only 15% of that obtained for Y-strain. The structure of sialoglycoproteins from *T. cruzi* Colombiana strain is under investigation.

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BC-50
CHARACTERISATION OF TWO GENES SPECIFICALLY EXPRESSED DURING *TRYPANOSOMA CRUZI* METACYCLOGENESIS

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Trypanosoma cruzi metacyclogenesis can be mimicked *in vitro* using chemically defined medium (Contreras et al. 1985 *MBP 16*: 315-327). During the first 6 hr of the process, there is a drastic diminution of the number of parasites in suspension, due to their adhesion to the culture flask. Infectious trypomastigotes will be generated through the differentiation of this population of adhered parasites, constituted of epimastigotes and of transition forms. The development of Representational Differential Expression (RDE) has already led to the cloning of *T.cruzi* stage-specific genes (Krieger & Goldenberg 1998 *Parasitol Today 14:* 163-166). The method consists in a selective amplification of genes specifically expressed by a given population (tester) following subtraction between this population and a reference population (driver). Here, we have used as tester DNA a population obtained by PCR amplification of cDNA synthesized from mRNA extracted from 6 hours of differentiation adhered epimastigotes, and as a driver an excess of DNA obtained from cDNA of exponentially growing epimastigotes, leading to the selection of two distinct clones whose expression is linked to differentiation. Clone #24 contains an open reading frame of 861 bp, coding for a protein with a predicted MW of 33.2 kDa. This protein shows 46% of identity (and 66% of homology) with a putative protein of *S. cerevisae*, whose function is still unknown. Southern blot analysis showed that this gene exists in two copies arranged in tandem, separated by a spacer of 3.4 kb. Northern blot analysis using polysomal RNA from parasites at various stages of the metacyclogenesis process showed a strong expression of gene #24 in the differentiating forms, but not in epimastigotes nor in trypomastigotes forms. Differently from clone #24, clone #88 exists in various copies in the genome of *T. cruzi*. Sequencing of some of these copies showed that the degree of conservation among them can vary from 75% to 98%. Several ORFs of small size could be derived from these sequences but none of them displayed any homology to sequences already existing in the Genebank. Northern blot analysis allowed the detection of the transcript of gene #88 at different stages of the metacyclogenesis process.

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BC-51
CHANGES IN EXTRACELLULAR ATP INDUCED CELL PERMEABILIZATION DURING ACUTE PHASE OF CHAGAS' DISEASE

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Chagas' disease is caused by the protozoan *Trypanosoma cruzi* and represents a public health problem in many developing countries. The P2Z purinoceptors are expressed by many lymphohematopoietic cells, including macrophages, microglial cells, mast cells, dendritic cells, thymocytes and some peripheral lymphocytes subsets. The physiological role of P2Z receptor is not yet well understood but it has been implicated in cell death, formation of giant multinucleated cells, maturation and release of IL-1 beta and, more recently, killing of intracellular mycobacteria. Herein, we examined the phenomenon of P2Z-induced permeabilization of splenocytes, thymocytes and intra-peritoneal cells of C57Bl/6 mice. Age-matched groups of animals were either infected with Y strain of *T. cruzi* or kept as uninfected controls. Cells were collected according to standard protocols, exposed to ATP at 37^{o}C in the presence of the fluorescent dye ethidium bromide and the degree of permeabilization was measured in a Beckton & Dickison FACSCALIBUR flow cytometer. During the acute phase of Chagas'disease (15 days post infection), the percentage of permeabilized cells from both spleen and peritoneal cavity was 30-40% higher than control ones. The dose-response curves of both ATP and BzATP displayed the expected patterns for P2Z purinoceptors. Moreover, UTP induced no permeabilization in the same group of cells. Preliminary phenotyping experiments performed by the simultaneous staining of surface markers with FITC-conjugated antibodies suggested that the increased ATP-induced permeabilization was displayed by both T lymphocytes and macrophages. In conclusion, our results show a possible modulation of P2Z purinoceptor during the course of this parasitic disease.

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BC-52
CAPTURE FOR SEQUENCING OF FRAGMENTS OF KDNA OF LOW REPRESENTATION AND HIGH INSTABILITY TANDEMLY INTEGRATED IN THE GENOME OF *TRYPANOSOMA CRUZI*-INFECTED HOST CELL

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The *Trypanosoma cruzi*-infected murine macrophage P388D1-IL1 were imortalized after eradication of the infection and cloning. These cell clones yield PCR and Southern blot signals, when kDNA primers and probes were used. Positive signals were not obtained with nDNA primers and probes. The PCR products showed sequences of minicircles of kDNA covalently integraded in direct and inverted tandem repeats in transposons of the LINE-1 family. Attempts of cloning and sequencing an integrated 2.3 kb fragment containing kDNA was precluded by its high instability in different vectors (lgt10, pBluescript, pCRII) and competent cells (*E. coli*, DH5a MRF', Sure cells and CES201). In every case, the plasmids that were examined showed rearranged kDNA stretches of not more than 600 bp. DNA instability, caused by unusual recombinations, have been described by several authors. In our Laboratory, this kind of rearranged DNA problem has been solved by a method of capture of the target sequences. We used specific linkers to combine with the stretches of genomic DNA, which had been digested with *Eco*RI and labelled with gATP^{32}. The radiolabelled sequences were then captured by hybridization with a 122 bp kDNA conserved region blotted in Biodyneâ. After several washings with 1X SSC for 15 min at 65 °C, the sequences that were eluted in distilled water for 1 h at 65°C, were concentrated in centricon-100â. PCR amplification of the sequences that had been captured was obtained with the linker sequence as primer. The amplification products that were each isolated after acrilamide gel electrophoresis (200 to 1300 bp), hybridized with the kDNA probe. The DNA fragments < 600 bp showed stability in DH5a. The sequencing of these clones revealed stretches of minicircles, whose structures were different from those that have been described for the *T. cruzi kDNA*.. The DNA fragments > 600 bp showed high instability in *E. coli*. A few plasmids showing inserts of expected size presented the linker in both ends. Their sequencing showed full conserved regions followed by truncated hypervariable regions of minicircles. Of interest, a 1169 bp clone showed 750 bp of the repetitive kDNA minicircle structure, as described above, followed by 419 bp of the murine host cell genome. The homology study has been confirmed by Southern hybridizations and, thus representing the flanking region of integration of kDNA into the genome of the host cell.

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BC-53
A TRYPOMASTIGOTE STAGE SPECIFIC C-DNA ENCODES THE LAMININ BINDING GLYCOPROTEIN (LBG) OF *TRYPANOSOMA CRUZI*

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Our group has previously shown that LBG, an acidic member of the Tc-85 family of trypomastigote surface glycoproteins binds to laminin. A full-length cDNA belonging to the Tc-85 family and recognized by monoclonal antibody H1A10 has also been cloned. This cDNA insert (named Tc85-11) has high sequence identity with all

previously described members of the gp85/trans-sialidase supergene family and hybridizes with several chromosome bands of the parasite genome, showing that several copies of Tc-85 genes are found in the *T.cruzi* genome. Laminin is a heterotrimeric glycoprotein belonging to a family of at least 11 described members. It is found in the basement membrane associated with collagen type-IV, entactin and heparan sulfate, forming a sheet surrounding organs and vessels. Since Tc85-11 encoded polypeptide has an expected pI of 5.00, we investigated whether such protein was LBG. The Tc85-11 coding region was subcloned into the pET21 expression vector, transformed into *E.coli* cells and metabolically labeled with ^{35}S-methionine. The bacterial cell lysate was added to either glutaraldehyde fixed LLC-MK_2 cells or laminin, fibronectin or gelatin coated wells. After an overnight incubation at 4^{o}C the wells were washed with flushing buffer and the bound radioactivity eluted with 1% SDS, counted and analyzed by SDS-PAGE and fluorography. Results have shown that the Tc85-11 polypeptide bound only to LLC-MK_2 cells and laminin, but not to fibronectin or gelatin. To confirm further that result, the Tc85-11 recombinant protein was purified from bacterial inclusion bodies, followed by Ni^{2+}/NTA-agarose chromatography and used to coat 96-wells microtiter plates. The wells were blocked with BSA, incubated with laminin, washed, and developed with anti-laminin rabbit sera (ELISA). It was found that laminin binds to the Tc85-11 polypeptide in a concentration dependent and saturable manner. These results show for the first time a specific ligand for one of the members of the Tc-85 glycoproteins in *Trypanosoma cruzi*. Together with other data in the literature showing that molecules with similar molecular mass and/or belonging to the gp85/transialidase family are able to bind to cells, the data herein presented reinforce the hypothesis that this family encodes glycoproteins with very similar sequences but differing enough as to bind to different ligands, and thus forming a family of adhesion glycoproteins which enables the parasite to overcome the barriers interposed by cell membranes, extracellular matrices and basal laminae.

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BC-54
A NEW MEMBER OF "CCHC" ZINC FINGER PROTEINS FAMILY FROM *TRYPANOSOMA CRUZI*

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Sequence specific single-stranded nucleic acid binding proteins have been described in many systems involved in processes such as transcriptional regulation, RNA processing and circular DNA replication. A distinctive feature of these proteins is the presence of CCHC class of zinc finger domains. Two proteins belonging to this family have been previously described in *Crithidia fasciculata* and *Leishmania major.* Degenerated oligonucleotides designed against CCHC domains were used to clone by PCR the homologous gene in *Trypanosoma cruzi.* The products obtained were subcloned and sequenced. Sequence comparisson analysis showed high degree of identity with other members of the family. Experiments suggest that there is more than one copy of this gene arrayed in tandem in the *T. cruzi* genome. Northern blot analysis showed expression in the epimastigote form of the parasite. Probable functions of this new protein and future experiments will be discussed.

BC-55
H NMR EVIDENCE OF SIALOSIDE HYDROLYSIS BY *TRANS*-SIALIDASE FROM *TRYPANOSOMA CRUZI* WITH RETENTION OF CONFIGURATION

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Trans-sialidase (TS) from *Trypanosoma cruzi*, the causative agent of Chagas' disease, preferentially catalyzes the transfer of sialic acid from Siaα2,3Galβ1,*x*-containing donor molecules to acceptors containing terminal β-galactopyranosyl residues. TS differs from mammalian sialyltransferases in that the latter use only CMP-NeuAc as donor substrate. Similar to viral and bacterial neuraminidases, TS also catalyzes a typical hydrolysis reaction. In fact, its amino acid sequence shows that *T. cruzi* TS belongs to the sialidase superfamily. Although there is only a moderate degree of primary amino acid sequence identity between these enzymes, the overall fold of the molecules and spatial arrangement of key amino acids at the active sites are remarkably similar. Furthermore, ^{1}H-NMR spectroscopy studies have clearly demonstrated that members of the sialidase family catalyze the enzymatic hydrolysis of the sialyl glycosidic bond with retention of configuration at the anomeric center of sialic acid. In order to determine whether *T. cruzi* TS has a topology and catalytic mechanism similar to other members of the sialidase family, we conducted an NMR investigation of the stereochemical course of the neuraminidase activity of recombinant TS from *T. cruzi* (a gift from Dr. Frasch, Univ San Martim, Buenos Aires). 4-Methyl-umbelliferyl-*N*-acetylneuraminic acid (4MU-NeuAc) and α2,3-sialyllactose were used as substrates, and the stereoselectivity of the enzymatic cleav-

age, at pH 5.8 and 37°C, was ascertained by monitoring the H3 axial and equatorial protons of the liberated sialic acid product during the course of the reaction. These ^{1}H NMR spectroscopic studies demonstrated that the thermodynamically less stable a-form of NeuAc is the first product of the cleavage. In the absence of acceptor, the formation of free a-NeuAc was followed by mutarotation, leading to an equilibrium mixture of the two isomers, the b-anomer being the major species at equilibrium. Our results also showed that 4MU-NeuAc is a significantly better substrate for the TS sialidase activity than a2,3-sialyllactose. In summary, we have shown that *T. cruzi* TS shares the stereoselectivity of its sialidase action with bacterial, viral, and mammalian sialidases, suggesting a similar active site architecture.

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BC-56
EVIDENCE OF SOME PROTEASES IN SHORT AND LONG EPIMASTIGOTES OF *TRYPANOSOMA RANGELI* BY ZYMOGRAPHIC TECHNIQUE

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The existence of intra-and extra-cellular proteases has been reported in different groups of Protozoa. About there is limited information concerning their presence in the Trypanosomatidae. Proteolytic enzymes of parasitic Protozoa have been implicated in a number of aspects of host-parasite interactions, including tissue and cell invasion, inactivation of deleterious host proteins, and catabolism of exogenous proteins for parasite metabolism. In the flagellate parasite, *Trypanosoma cruzi*, a number of different protease activities have been described, using a variety of different detection methods. Several distinct protease activities are expressed during *T. cruz*i differentiation and some of these proteases are developmentally regulated. A distinct alkaline cysteine protease has been detected in *T.cruzi* and other trypanosomatids. Lowndes et al., in 1996, showed the expression of protease during *T.cruzi* metacyclogenesis (transformation of epimastigotes into metacyclic trypomastigotes). In *Leishmania* spp a number of proteases associated with the cell surface of promastigotes have also been characterized. In our work, we investigated proteases in *T. rangeli* strain H14 when cultures were maintained at 28°C in NNN and LIT medium supplemented with 20% heat-inactivated foetal calf serum. Epimastigotes synchronously reproduced in culture exhibiting long and short forms during differents days of cell growth and were used in all experiments. We used a Zymographic technique for detection and partial characterization of protease from long and short forms with SDS-polyacrilamide gel electrophoresis (SDS-PAGE) incorporating 0.1% gelatin into the gel as a substrate. For protease characterization, the same procedure will be carried out with different classes of proteolytic enzymes inhibitors.

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BC-57
THE *LEISHMANIA* SPLICED LEADER SEQUENCE BEHAVES AS A TRANSLATIONAL ENHANCER IN THE RABBIT RETICULOCYTE TRANSLATION SYSTEM

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All mRNAs in Trypanosomes are generated through *trans*-splicing. The 39nt 5' terminal exon (the spliced leader, SL) and its unique cap4 structure is transferred from a SL RNA precursor to the 5'end of the pre-mRNAs. The two independently transcribed RNA sequences are joined and the policistronic primary transcript is therefore processed generating the mature monocistronic mRNAs. As a result, the same short, capped, non-coding leader sequence is present at the 5'end of each mRNA. A t*rans* spliced 5' leader sequence has also been identified in most but not all nematode mRNAs. The nematode 22nt SL sequence was found to functionally enhance translation in a message-dependent protein synthesis system obtained from nematode embryos. In trypanosomes very little is known about the role of the SL sequence in mRNA metabolism as well as its effect on translation. This work proposes to study the effect of the *Leishmania* SL sequence on the translation of two different *in vitro* transcribed reporter RNAs: those coding for frog Cyclin A and human Poly(A) Binding Protein (PABP). Oligonucleotides were designed based upon published *Leishmania* SL sequences and they were cloned downstream of the T7 promoter of the pTZ18R transcription vector (Pharmacia), followed by the coding sequences of either PABP or Cyclin A. Transcription was then performed with T7 RNA polymerase in the presence/absence of m^7GTP Cap. Resulting mRNAs had 7 nt between the 5'end and the beginning of the SL sequence. They varied as to the coding sequence and the presence/absence of either the Cap, SL sequence and a poly-A tail. These mRNA were then tested for translation in the message-dependent rabbit reticulocyte lysate. Maximum translation was obtained with mRNAs containing both Cap and the SL sequence, whilst the presence of the poly(A) tail did not produce any significant effect. Similar results were seen for both Cyclin and PABP indicating that the stimulatory effect by the SL sequence is independent

of downstream sequences. The presence of the Cap was not required for this effect but they appear to act synergistically in stimulating translation. In summary our data provide evidence that *Leishmania* SL sequence can behave as a translational enhancer *in vitro* in a system derived from a phylogenetically distant organism. It suggests that the machinery required for recognizing the SL signal is evolutionary widespread and conserved despite the SL sequence and *trans*-splicing be restricted to only a few groups of eukaryotes.

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BC-58
THE DETECTION OF LEISHMANIAL PARTIAL SSU RRNA FRAGMENT IN THE TISSUES OF WILD ANIMALS CAPTURED IN AN ENDEMIC AREA OF CUTANEOUS LEISHMANIASIS IN 'ZONA DA MATA' OF THE STATE OF PERNAMBUCO, BRAZIL

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A total of 19 spleen biopsies were obtained from wild animals that had been captured in an endemic area of cutaneous leishmaniasis in the State of Pernambuco, Brazil. So far all the parasites that have been isolated from man in the region belong to one species, *Leishmania (Viannia) braziliensis.* The tissue samples from each animal were frozen at -196^{o}C and subsequently thawed for DNA extraction. The polymerase chain reaction (PCR) was performed at an annealing temperature of 53^{o} C using oligonucleotides that amplify the 3' end of the 18S region (Uliana et al., 1994. J Euk Microbiol 41:324-330). The PCR of samples from three animals (*Rattus frugivorus*, *Rattus alexandrinus*, *Marmosa* sp.) produced the expected fragment. The two rats had been captured in banana and sugar cane plantations and the marsupial was from a small piece of residual primary Atlantic rain forest. The PCR amplification of material from a further six samples (1 *Rattus frugivorus* , 4 *Bolomys lasiurus* and 1 *Nectomys squamipes*) resulted in the expected fragments but other non-specific fragments were also generated.

The PCR results suggest that the incidence of *Leishmania* infections in wild animals in this particular endemic area is high.

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BC-59
TARGETING OF CYSTEINE PROTEINASES IN *LEISHMANIA PIFANOI*: EVIDENCE FOR CONSERVATION OF TARGETING SIGNALS BETWEEN SPECIES AND ROLE OF GLYCOSYLATION IN THE MATURE DOMAIN

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Amastigote-specific cysteine proteinases of the *Leishmania mexicana* complex are located in lysosomal organelles called megasomes. We are presently studying the targeting mechanisms of these hydrolases to the lysosome in *Leishmania*. Following their synthesis in the rough endoplasmic reticulum, eukaryotic lysosomal proteins are transported to the Golgi complex, where they are sorted from molecules bound to the cell surface, and subsequently directed to the lysosomes. Mannose 6-phosphate serves as a signal for targeting proteins to the lysosome in mammals but the role of asparagine linked carbohydrate in yeast and trypanosomatid vacuolar proteins remains unclear. To study the possible implication of cysteine proteinase glycosylation on cellular targeting, we performed site-directed mutagenesis in the abundant *L. pifanoi* cysteine proteinase gene (*lpcys2*), containing the epitope tag AU1 at the C-terminus. The mutations were introduced through PCR at the coding sequence for the asparagine residues potentially glycosylated present in the mature proteinase, C-terminal domain and in both simultaneously. The mutated genes were cloned into the transfection vectors pX63-neo and pG1 and these constructs transfected into both *Leishmania major* and *L. pifanoi*. An *L. major* transfectant has already been characterized by immunoelectron microscopy, using a polyclonal antibody against Lpcys2. The control, non-transfected parasites, showed no labelling, indicating no cross-reaction with the endogenous proteinases. Both transfectants containing the non-mutagenized gene or the gene without the potentially glycosylated asparagine in the mature domain, showed intense labeling in electron-dense vacuoles compatible with lysosomes. This indicates the recognition of lysosomal targeting signals across species in *Leishmania* and the apparent absence of a role for the mature domain glycosylation in targeting. We are presently characterizing the *L. major* and *L. pifanoi* transfectants containing the mutations in the C-terminal extension and in both domains.

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BC-60
STUDIES FOR GENETICS MANIPULATIONS IN *LEISHMANIA (VIANNIA) BRAZILIENSIS*

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Adequate protective vaccines against *Leishmania* infections are not developed yet, and drugs currently available for chemotherapeutic intervention are mostly unsatisfactory. In *Leishmania* the enzymes Dihidrofolate Reductase Thymidilate Synthase (DHFRTS) and Trypanothione Reductase (TR) are important to metabolism of folates and thiols respectively. The bifunctional enzyme DHFRTS has critical roles in intermediary metabolism. TR is a flavoprotein disulphide reductase that catalyse the NADPH-dependent reduction of trypanothione. Both enzymes DHFRTS and TR are potential targets for chemotherapy of trypanosomatid infections. Although *Leishmania (Viannia) braziliensis* represent a serious health problem in South America, relatively little experimental work has been done to characterise this parasite. The objective of the present study is to optimise conditions and obtain null mutants for *DHFRTS* and *TR* by gene replacement in *L. braziliensis* using selectable markers. Transient transfection experiments were carried out successfully. A genomic library of *L. braziliensis* (LB 2904 strain) was constructed in cLHYG vectors (Ryan *et al.* Gene, 131:145-150, 1993). Genomic clones carrying *DHFRTS* and *TR* genes were rescued from the library and the analyses of their structures are currently being made. Have been shown that promastigote forms of *L. tropica* selected by resistance to the antifolate MTX overproduce DHFRTS: a 30 kb segment of DNA (the R region) is amplified in the form of an extrachromosomal circular DNA in antifolate-resistant cells. *L. braziliensis* was cultured in the presence of 0.1, 0.5 and 1.0 mM MTX but MTX-resistant cells were not obtained.

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BC-61
STRUCTURAL CHARACTERIZATION OF THE RIBOSOMAL DNA INTERGENIC REGION OF *LEISHMANIA TARENTOLA*

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A construct encompassing approximately 3Kb of the IGS/ETS region of *Leishmania tarentolae* rDNA showed the ability of driving the expression of bacterial chloramphenical acetyltransferase (CAT) when transfected into the homologous species and into other *Leishmania* species (Orlando et al. 1997 *Mem Inst Oswaldo Cruz 92*(Suppl): 176). This fragment has been further characterized. The restriction enzyme map and the entire nucleotide sequence were obtained and compared to the corresponding sequence of *L. amazonensis* (GenBank accession number U21687), *L. chagasi* (U42465) and *L. donovani* (L38572), showing a high degree of sequence identity. The transcription start point was mapped at 976bp from the 5' end of the 18S rRNA gene by Northern blot and primer-extension experiments. In the IGS region, we found 15 to 18 blocks of an element 63bp long. No similarity was detected between this repeated element and those found in the other *Leishmania* species. The repeats initiate 214bp upstream to the transcription start point. We also found two copies of another element, 160bp long, upstream to the first repeated block. This element can potentially form a steam loop structure similar to that described for the bacterial rho-independent transcriptional terminator binding site. This element is also very similar to a sequence described downstream to the 3'end of the *L. infantum* rRNA gene that was shown to have a trancriptional terminator functional role (Requeña et al. 1997 *Mol Biochem Parasitol 84*: 101-110). Since the spacer sequence evolves at a faster rate than the coding region and could be more suitable for studies in very closely related organisms, characterizing the structure of the intergenic region, will also provide the means to use the sequence data as a molecular marker to infer the phylogenetic relationships amongst *Leishmania* species.

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BC-62
STRATEGIES FOR MOLECULAR CLONING AND SEQUENCING OF PROGRAMMED CELL DEATH PUTATIVE GENES IN *LEISHMANIA (LEISHMANIA) AMAZONENSIS* PROMATIGOTES

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Programmed cell death is a gene-regulated phenomenon and was thought to be exclusive of higher eukaryotic cells. In spite of this established concept, we have shown that protozoan parasites, *Leishmania (Leishmania) amazonensis* promatigotes, die by apoptosis when submitted to an *in vitro* heat shock in the presence of calcium ions. We are presently looking for mammalian homologue genes responsible for this type of death in these parasites. To this end promatigotes were submitted (incubated at 34ºC/37ºC for 4 hr) or not (incubated at 22ºC for 4 hr) to stressing conditions and had their mRNA extracted. The cDNA further synthesized was used as a template for RT-PCR. Conserved motifs of the caspase family of ICE/CED-3-related cysteine proteases and of the Bcl-2 protein family were used to construct nested primers. These gene families products display major roles in the modulation of programmed cell death in higher eukaryotic cells. We have also used the 35-nucleotide spliced leader sequence common to the 5'-end of all trypanosomatid mRNAs to design two other oligonucleotides. Four different protocols were established and eight bands with molecular size ranging from 0.3 to 1.0 kb were extracted from 1% agarose gels. These DNAs are being cloned in pCR® II-TOPO vector (Invitrogen) and automatically sequenced with the Taq Dye Primer Cycle Sequencing Kit (Applied Biosystems). We believe the knowledge of modulatory mechanisms of promastigotes death, in similar conditions they face in the very moment of infection of their vertebrate hosts, is a crucial step for understanding the establishment of leishmanial infection.

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BC-63
SEARCHING FOR CENTROMERIC SEQUENCES OF *LEISHMANIA MAJOR*

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The centromere is a specialized region of the eukaryotic chromosome that is the site of kinetochore formation and spindle attachment and of regulation of chromosome movement during mitotic and meiotic cell divisions. An evaluation of the available data from yeast to man suggests that, although centromeres are highly variable DNA elements, a conserved pattern of function occurs.

In order to localize and characterize discrete centromeric regions in *Leishmania* we are utilizing 3 different libraries constructed in linear vectors. These are a special kind of vectors, which can be linearized by digestion with the restriction enzyme *Swa* I, generating a linear molecule bearing in both extremities telomeric repeats of *T. brucei*. They look like an artificial chromosome inside the parasite cell, so we denomined LAC - *Leishmania* artificial chromosome. These libraries are; (i) a complete genomic library; (ii) a library representing the chromosomal band of 320 kb (chromosomes 2 and 3) and (iii) a library representing the chromosomal band of 500 kb (chromosome 6). From the complete genomic library a set of 50 clones had been limited to 2 clones which, for their dinamic of maintenance of exogenous molecule, are still under test. From each of the subgenomic libraries, one hundred clones were rescued and thirty-six of them (shown to be different from each other) have been pooled in-groups of 6 to be transfected into *L.major* LT252. Currently, sixty clones are under analysis: they are kept under no drug pressure and tested for maintenance of LACs.

The large number of transfectants under analysis, led us to design strategies to make the task feasible and to shorten the period needed to follow each transfectant. The first one is a modification of the dilution limiting assay and the second is the use of a system of replica plating a large number of clones further analyse the growth with and without drug in Elisa reader. The whole strategy, potentially useful in a number of other studies and applications, will be presented.

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BC-64
REGULATION OF PROLIFERATION OF *LEISHMANIA AMAZONENSIS* BY NUCLEOTIDES: ROLE OF ECTO-ENZYMES

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Plasma membrane of cells contains enzymes whose active sites face the external medium rather than cytoplasm. The activities of these enzymes, referred to as ecto-enzymes, can be measured using intact cells and can be modulated by divalent cations such as Mg^{+2} and Ca^{+2}. It has been shown that ATP and other nucleotides stimulate the proliferation of different cell types, while the inhibition of the ecto-ATPase, 5'nucleotidase, and phosphatase activities diminish the stimulatory effect of ATP (Lemmens et al. 1996 *Biochem J 316*: 551-557). We have recently characterized a Mg-dependent Ecto-ATPase in *Leishmania tropica* (Meyer-Fernandes et al. 1997 Arch Biochem

Biophys *341:* 40-46). In the present study we show that AMP and ADP but not ATP inhibit *Leishmania amazonensis* cell proliferation in a dose-dependent manner. In addition we showed that *L. amazonensis* present 5 different ecto-phosphomonoesterase activities. These cells are able to hydrolyze ATP, ADP, 3'AMP, 5'AMP and *p*-nitrophenylphosphate (*p*-NPP). Cellular viability was assessed, before and after incubations by mobility and trypan blue methods. The activities were linear with time until at least one hour (phosphatase activity = 260 nmol Pi/h/10^7 cells, 3'nucleotidase = 55 nmol Pi/h/10^7 cells, ATPase = 20 nmol Pi/h/10^7 cells, ADPase = 15 nmol Pi/h/10^7 cells, 5'nucleotidase = 9 nmol Pi/h/10^7 cells.The ecto-ATPase here described is stimulated by $MgCl_2$ and $MnCl_2$ but not by $CaCl_2$ and $SrCl_2$. The ADPase, 5'nucleotidase, 3'nucleotidase and phosphatase activities are not stimulated by $MgCl_2$.

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BC-65
REACTIVITY OF *LEISHMANIA MAJOR* AMASTIGOTE GLYCOLIPIDS WITH THE MONOCLONAL ANTIBODY MEST-1. STUDY OF A POSSIBLE ROLE OF GALACTOFURANOSE IN THE MACROPHAGE-PARASITE INTERACTION

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Recently, it was demonstrated that monoclonal antibody (MoAb) MEST-1 was able to recognize terminal residues of b-D-galactofuranose of parasite glycolipids such as GIPL-1 from *L. major* promastigotes and LPPG from *T. cruzi* epimastigotes (1997 *Glycobiology 7*: 463). MoAb MEST-1 was able to detect as low as 1 ng of GIPL-1, by solid-phase radioimmunoassay, and it was verified that this glycolipid is the only antigen recognized by this MoAb in promastigote forms of *L. major*. By HPTLC immunostaining with MEST-1, it was confirmed the presence of GIPL-1 in amastigote forms of *L. major* isolated from hamster footpad lesions. It was observed a lower concentration of GIPL-1 in amastigotes than in promastigotes. This data is in agreement with recent immunofluorescence results, where it was detected a lower fluorescence for amastigotes in comparison to that observed to promastigotes. By HPTLC immunostaining, Western blot, and indirect immunofluorescence no cross reactivity of MEST-1 was observed with amastigote and promastigote forms of *L. (L.) amazonensis*, *L. (V.) braziliensis* and *L. (L.) chagasi*. Infectivity assays showed that MEST-1 Fab fragments are able to inhibit 30% and 80% of macrophage invasion by amastigote and promastigote forms, respectively. These results indicate that GIPL-1, present in both amastigote and promastigote forms, is involved in the *L. major*-macrophage interaction.

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BC-66
PURIFICATION AND PARTIAL CHARACTERIZATION OF PROTEASOMES FROM *LEISHMANIA AMAZONENSIS*

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In spite of the fact that eukariote proteasomes have been implicated in several cellular functions - such as the cell cycle control and proteolysis of abnormal proteins - there are few studies on the role of this macromolecular proteinase in protozoan organisms. We have previously shown in *Trypanosoma cruzi* that, in axenic cultures, the inhibition of the proteasome activity with lactacyctin interferes with the transformation of trypomastigotes into amastigotes. The inhibition of the parasite remodeling was considered to be directly dependent on the blockage of proteolysis. Here, we report the isolation and partial characterization of proteasomes from *Leishmania amazonensis* and the effect of the inhibition this multicatalytic protease on the replication of the parasite. Cytoplasmic extracts of *L. amazonensis* promastigotes were prepared and submitted to liquid chromatography on FPLC. Through gel filtration and anion exchange, a major peak of about 670 kDa was isolated. The proteasome activity was monitored based on the property of lactacystin to specifically inhibit the proteolytic activity of proteasomes, using fluorogenic peptides. This peak contains at least the two classical proteolytic activities of the proteasomes: trypsin-like and chymotrypsin-like activities. The trypsin-like activity was higher than the chymotrypsin-like activity and, as in mammalian proteasomes, it was less sensitive to inhibition by lactacystin than the chymotryptic activity. Neither activities was inhibited by E-64, PMSF, *o*-phenanthroline or pepstatin-A. Silver staining of the purified material, after SDS-PAGE

under denaturing conditions, revealed the typical band pattern for proteasomes: a distribution of molecular weights ranging from 25 to 35 kDa. We have found that the addition of lactacystin to the culture medium of *L. amazonensis* promastigotes produces, in a dose-dependent fashion, inhibition of parasite cell division. *L. amazonensis* promastigotes that grow in the presence of 50mM lactacystin remain viable up to eight days in culture, indicating that although the proteasome activity is required for parasite replication, it is not essential for their survival.

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BC-67
PREPARATION OF SYNTHETIC PROBES TO TEST ACCEPTOR SUBSTRATE SPECIFICITY OF THE ELONGATING a-D-MANNOSYLPHOSPHATE TRANSFERASE IN *LEISHMANIA*

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The phosphoglycan part of the surface antigenic lipophosphoglycan produced by the promastigote form of all *Leishmania* species is a polymer consisting of (1AE6)-linked b-D-Galp-(1AE4)-a-D-Manp-phosphate repeating units and containing a mannobiosyl phosphate cap at the nonreducing end. The 3-OH group of D-galactose can be randomly substituted with mono-, di-and trisaccharide fragments. Biosynthesis of the disaccharide phosphate backbone has been studied in vitro using a crude mebrane preparation from *L. major* promastigotes, GDP-[3H]Man and synthetic phosphooligosaccharides 1-6 (fragments of *Leishmania* phosphoglycan backbone) as exogenous acceptors. The oligomers 1, 3 and 4, containing a terminal galactosylmannosyl phosphate structure, were shown to be essentially equally efficient as acceptors for a transfer of the a-D-[3H]Manp-phosphate moiety to the b-D-galactosyl residue with an a-D-mannosyl phosphate transferase (MPT) present in the *Leishmania* membranes. In contrast, the galactosylmannoside 2, which does not contain the phosphate, was a poor acceptor, neither compounds 5 and 6, containing the terminal mannobiosyl phosphate cap. Thus the minimum exogenous acceptor structure for the MPT activity detected in the membrane preparation is the phosphodisaccharide 1. A set of novel phosphodisaccharide substrate analogs 7-13 then was designed and synthesized chemically. Compounds 7-13 differ from 1 either by the opposite configuration of the specific carbon atoms in a-D-Manp (C-2 in 7 or C-3 in 8) and b-D-Galp (C-2' in 10, C-3' in 11 or C-4' in 12) residues, or by the deoxygenation of C-6 (in 9) or C-6' (in 13) atoms. The information obtained from testing of the acceptor capacity of 1 and 7-13 will be used to determine the acceptor substrate specificity of the MPT in *Leishmania* (which sugar hydroxyl groups are involved in enzyme-substrate interaction) and to design potential enzyme inhibitors.

BC-68
POSSIBLE ROLE OF INOSINE-5'-MONOPHOSPHATE DEHYDROGENASE IN APOPTOSIS OF *LEISHMANIA (LEISHMANIA) AMAZONENSIS* INDUCED BY HEAT SHOCK

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In the last few years, parasitic diseases have grown and spread world-wide. The main factor responsible for their establishment is the ability of parasites to scape from host immune system. Since then many enzymes have been characterized in order to provide chemotherapeutic treatments. We intend to clone, sequence and analyse the expression of one of these enzymes: Inosine-5'-monophosphate dehydrogenase (IMPDH) of *Leishmania amazonensis*, the ethiologic agent of leishmaniasis. IMPDH plays a major role in the salvage pathway of purine metabolism in Trypanosomatidae. A hallmark of leishmaniasis natural history is the heat shock suffered by promastigotes when inoculated in their hosts by the insect vectors (Phlebotominae family). We have previously shown that 20% of the parasites die by apoptosis when submitted to an *in vitro* heat shock in the presence of calcium ions. Considering that an IMPDH inhibitor (Tiazofurin) promotes apoptosis in 100% of a tumoral cell line, we decided to investigate IMPDH expression in parasites submitted (incubated at 34^{o}C with FCCP - a mithocondrial uncoupler) or not (incubated at 22^{o}C, their optimal growth temperature) to stressing conditions. Both samples had their mRNA extracted and the cDNA further synthesized was used as a template for RT-PCR. We found a 359 bp fragment that displays an identity of 94,3% with *L. donovani* IMPDH. Using this fragment as a probe, we are cloning and sequencing total *L. amazonensis* IMPDH gene. Furthermore, the expression of IMPDH is currently under investigation in promastigotes treated as described above.

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BC-69
PHYSICAL MAPPING ACROSS *LEISHMANIA MAJOR* GENOME

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The objetives of this work are to test a strategy for the production of a low-resolution physical map of the genome of *Leishmania major* (LV39, Rho/Su/59/P) using a direct probe hybridization technique and to construct concomitantly a transcriptional map. The approach should reduce time and costs required for the completion of a physical map. We have centered our efforts on a random mapping approach for the construction of contigs. We have used 358 ESTs (Expressed Sequence Tags, generated from a promastigote LV39 cDNA library at the laboratories of J. Blackwell - Cambridge, UK - and H. Schneider - UFPA, Brazil) to rescue genomic clones from the *L.major* genomic library and assign the markers to chromosomal bands. We have also installed and organized a system for data storage and analysis, using FoxPro and Excel, which allowed the contig assembly by Sam (a contig assembler software developed at Sanger Centre, UK). The designed strategy of storage, data analysis and assembly by Sam has been successful and several contigs have been confirmed through restriction profile and hybridization analysis. To better analyze some map inconsistencies we are currently working with Segmap (a contig assembler software developed by C Magness and P Green). The reorganization of data to the Segmap format is in progress. As part of our strategy for the construction of an associated transcriptional map of *Leishmania*, a promastigote cDNA library (Levick et al. 1996 *Mol Biochem Parasitol 76*: 345) has been pooled to be used as a tool to increase markers density onto contigs. Pools of 24, 48 and 96 clones were built and transferred to nylon membranes. The same pools were also submitted to a round of amplification prior to membrane transfer. Cosmid clones placed in the contigs were used to rescue cDNA sequences present in the pools. Only amplified pools of 24/48 clones gave consistent results; data will be presented. Analysis of the collected data, including general aspects of EST assignment specificity and sequence features as well as distribution of genes in the contiged regions will be presented.

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BC-70
PCR ANALYSIS OF LEISHMANIAL BIOPSIES NEGATIVE BY TRADITIONAL DIAGNOSTIC METHODS

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American Tegumentary Leishmaniasis (ATL) caused by *Leishmania (Viannia) braziliensis* is mainly presented as chronic cutaneous ulcers that may heal spontaneously (LCL) and has the possible hazard of late development of mucosal lesions (MCL). Diagnostic procedures for ATL are based mostly on i) clinical presentation, ii) epidemiological history, iii) parasitological tests such as touch preparation (TP) and in vitro culture of lesion material (biopsy or needle aspirate), iv) Montenegro skin test and, as a last resource, histopathological analysis with regular staining (H&E) or immunoperoxidase. These tests are often time consuming, cumbersome and lack sensitivity, specially when dealing with *L. braziliensis* infections, due to the scarcity of parasites within the lesions. In an attempt to circumvent the traditional difficulties regarding ATL laboratory diagnosis, the Polymerase Chain Reaction (PCR) methodology was recently introduced. In order to evaluate the PCR technology, 41 leishmanial biopsies that were negative by traditional methods, were subjected to amplification of a mitochondrial DNA fragment of the parasite. Biopsies have been taken from the border of the lesions as a routine for diagnosis and each specimen has been divided into fragments for conventional methods (H&E, culture in Schneiders medium and TP). As they all showed to be negative by these techniques, 30 sections of 5 mm thickness of the paraffin-embedded tissue were cut with a microtome, using a disposable blade for each sample. Paraffin was removed by extraction with xylene, and DNA was purified by column chromatography (QIAamp Tissue Kit, QIAGEN) following the manufacturers instructions. The DNAs were ethanol precipitated and resuspended in 10 ml of TE (10 mM Tris-HCl pH 8,0; 1 mM EDTA pH 8,0). Aiming to amplify the conserved region of the minicircle molecule, oligonucleotides [5'(G/C)(G/C)(C/G)CC(A/C)CTAT(A/T)TTACACCAACCCC & 5'GGGGAGGGGCGTTCTGCGAA] were used promoting the amplification of a 120 bp product. The products were analyzed by agarose gel electrophoresis and ethidium bromide staining. The PCR results in the 41 biopsies that were negative by conventional diagnostic methods, showed 78% of positivity. The advent of a highly sensitive molecular approach such as PCR allowed the detection of parasite DNA in clinical samples that showed to be negative by traditional methods. Hybridization experiments with a *Viannia* specific probe are being carried out in order to type the species involved and also to enhance the sensitivity of the assay.

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BC-71
PARTIAL CHARACTERISATION OF DIHYDROXYACETONEPHOSPHATE ACYL-TRANSFERASE OF *LEISHMANIA MEXICANA* PROMASTIGOTES

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Previously, we have identified all five enzymes involved in the initial steps of ether-lipid biosynthesis associated with glycosomes of *Leishmania mexicana* promastigotes (Hart & Opperdoes 1984 *MBP 13*: 159-172, Heise & Opperdoes 1997 *MBP 89*: 61-72). Dihydroxyacetonephosphate acyl-transferase (DHAP-AT) is one of the first enzymes in the pathway: it catalyses the acylation of DHAP using acyl-CoA as the fatty-acid donor. The reaction is totally dependent on acyl-CoA and partially dependent on the presence of NaF, $MgCl_2$ and BSA. The optimal conditions were obtained using 0.15mM palmitoyl-CoA/0.3mg BSA ratio in each assay. Replacement using myristoyl-CoA or stearoyl-CoA caused strong inhibition. The specific activity of DHAP-AT in purified glycosomes was 20 fold increased relative to a total cell extract. Kinetic analysis have shown a K*m* for DHAP of 0.307mM (sd: 0.030) and a V*max* of 1050 pmol/min/mg of protein (sd: 67). The K*m* for palmitoyl-CoA was difficult to measure since at higher concentrations of palmitoyl-CoA the reaction displayed non-Michaelis-Menten kinetics. All the DHAP-AT activity was associated with the membrane fraction of purified glycosomes and pH curves indicated a broad pH optimum ranging from 6.4 up to 8.0. The optimal activity was dependent on the type and molarity of the buffer system used. Addition of very low quantities of several ionic and non-ionic detergents strongly inhibited DHAP-AT. The zwitterionic cholate derivative CHAPS was shown to preserve DHAP-AT up to the concentration of 0.2%. However, it was unable to solubilize the majority of the enzyme in an active and stable form. After solubilization, DHAP-AT activity was lost and was not recovered even after membrane reconstitution experiments. Assays performed *in vitro* incubating purified glycosomes in the presence of high concentrations of the leishmanicidal ether-lipid analogues Miltefosine and Edelfosine had shown no inhibition of DHAP-AT or alkyl-DHAP synthase activities. These results suggested that the analogues do not inhibit early-steps of the ether-lipid biosynthesis in *Leishmania mexicana*.

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BC-72
PARTIAL CDNA CLONING OF A GENE ENCODING FOR A 120 KDA COLLAGEN-BINDING PROTEIN FROM *LEISHMANIA MEXICANA*

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Once inoculated into the skin, *Leishmania* parasites are exposed to cellular and extracellular matrix components such as collagen, elastin, fibronectin, glycosaminoglycans and proteoglycans. We have previously reported that *L. mexicana* promastigotes interact with type I collagen in a dose dependent manner (1997 *Exp Parasitol 85*:149), suggesting that parasite cell membrane receptor(s) may confer tropism for the skin. In order to study the role that collagen receptor(s) could play in the pathology of the disease, two collagen-binding proteins have been purified by affinity chromatography from promastigote extracts. Polyclonal antibodies against these proteins, 120 and 18 kDa, revealed that they are localized on the parasite surface. To clone the genes that codify for those proteins, a cDNA library from *L. mexicana* constructed in λZAP-II was screened with the polyclonal antibodies. One clone (pB351) out of 7 positives was selected for further characterization. An insert of 1.8 kb was present in this clone, and immunoabsorption and Western blot assays, showed that the clone partially codifies for the 120 kDa protein. The DNA sequence showed homology with proteins involved in the recognition of extracellular matrix components by eukaryotic cells (integrins). The DNA insert has been cloned in an expression vector in order to purify the recombinant peptide and to produce polyclonal antibodies in experimental animals. After getting the antibodies, we will conduct some functional assays with *Leishmania* parasites. *Leishmania* parasites face up a strong pressure selection induced by distinct microenvironments they can infect. Our aim is to define the role the 120 kDa protein that binds to collagen could play in the parasite biology and to determine its participation in the pathogenicity process of *Leishmania* parasites.

BC-73
N-LINKED OLIGOSACCHARIDE STRUCTURES OF THE FML ANTIGEN OF *LEISHMANIA (L.) DONOVANI*

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The Fucose Mannose Ligand (FML) is a complex glycoprotein fraction present on the surface of pro and amastigotes of *Leishmania (L.) donovani*, that strongly inhibits the *in vitro* macrophage infection by both forms of the parasite. The analysis of FML sugar molar composition disclosed the presence of: ribose (16.8%), fucose (5.3%), xylose (12.6%), mannose (38.5%), galactose (3.3%) glucose (13.7%), *N*-acetyl glucosamine (7.4%) and NANA (0.2%). In previous work we described the isolation of the *N*-linked oligosaccharides of the FML antigen of *L.(L.) donovani*, their separation by HPLC, gel permeation chromatography and their characterization by chemical analysis. The FML fraction was obtained according to Palatnik et al. (1989 *Infect Immun 57*: 754). The oligosaccharides were released from FML by hydrazinolysis (Bayard & Fournet. 1975 *Carbohyd Res 46*: 75). These conditions separe the glycidic from the protein moieties of N-linked glycoproteins. The resulting oligosaccharides were *N*-reacetylated according to Reading (1978 *J Biol Chem 253*: 5600) and further reduced with $NaBH_4$. The oligosaccharides were subjected to HPLC on an 5mm RP-18 column using 500mM potassium dihydrogen phosphate (KH_2PO_4) as eluent giving 15 fractions. The methylated derivatives were identified and quantitated by GLC-MS. The major fraction (F5) corresponding to 33.5 % of the FML *N*-linked glycidic moiety, purified by Bio-Gel P-2 column chromatography was shown to composed of linear oligossacharides of 4-*O* (42%), 3-*O* (22%) linked Man*p*, 2-*O*-(3%) linked Fuc*p*, 2-*O* (tr) linked Man*p* in trace amounts and Gal*p* as terminal units. In this work a second oligossacharide was characterized by the same methodology (F4). It corresponds to 20.3% of the FML *N*-linked glycidic fraction. GLC analysis disclosed the presence of Man (52.3%), Gal (26.2%), Fuc (4.1%) and GlcNac (18.4%). Methylation analysis of this fraction gave residues of galactopyranose (2,3,4,6, Me_4-Gal, 33%), manopyranose (2,3,4,6, Me_4-Man 8%, 2,3,6 Me_3-Man 12%, 2,4,6 Me_3-Man 6%), fucopyranose (2,3,4-Me_2-Fuc 4%) and GlcNac (2,3,6, Me_3-GlcNac 35%, 2-Me-GlcNac 2%) which were identified and quantitated by GLC-MS. This FML *N*-linked second major fraction is a branched oligossaccaride composed of 4-*O* and 3-*O* Man*p and* 4-*O* linked GlcNac alternating units with GlcNac as a branching point and Gal*p*, Man*p* and Fuc*p* as terminal residues. The structure and of F4 was confirmed by H^1 Nuclear Magnetic Resonance (^{1}HNMR) Spectroscopy. The macrophage Mannose-Fucose receptor (MFR) has been pointed out as one of the major mediators of *L. (L.) donovani* penetration. The branched structure of F4 fraction exposing mannopyranose and fucopyranose residues, could explain the strong inhibitory potential of the FML antigen on murine macrophage infection by promastigotes and amastigotes of *L.(L.) donovani* (Palatnik et al.1989 *loc. cit.).*

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BC-74
MONOCLONAL ANTIBODIES AGAINST LPG OF *LEISHMANIA (L.) AMAZONENSIS*

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Two monoclonal antibodies (MoAbs) VST-1 (IgG3) and VST-2 (IgM) direct to LPG were recently produced after immunization of BALB/c mice with purified LPG fraction of *Leishmania (L.) amazonensis*. The MoAbs were characterized by indirect immunofluorescence, solid-phase radioimmunoassay and Western blot. The reactivities of these antibodies to promastigote forms of *L. (L.) amazonensis*, *L. (L.) major, L. (V.) braziliensis* and *L. (L.) chagasi* were analyzed. MoAbs VST-1 and VST-2 showed to react only with promastigote forms of *L. (L.) amazonensis* and *L. major*, more specifically with the purified LPG fractions. VST-1 and VST-2 reactivities were abolished upon mild oxidation with sodium meta-periodate of LPG molecules, indicating that these antibodies recognize carbohydrate epitopes. As reported previously MoAb ST-3 recognizes only LPG molecules of promastigote forms of *L. (L.) amazonensis* and is able to inhibit about 80% of macrophage invasion by the parasite. On the other hand VST-1 and VST-2 recognize LPG of promastigote forms of *L. (L.) amazonensis* as well as LPG from promastigotes of *L. major*, therefore it is reasonable to infer that the epitopes recognized by VST-1/VST-2 and ST-3 in LPG molecules are distinct. The fine specificity of VST-1/VST-2 as well as their ability to inhibit binding of parasites to macrophage are currently under investigation.

Supported by Fapesp, CNPq, and Pronex.

BC-75
MONOCLONAL ANTIBODIES SPECIFIC TO *LEISHMANIA (VIANNIA) BRAZILIENSIS*

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Monoclonal antibodies (MoAbs) to *Leishmania (Viannia) braziliensis* (serodeme 1) were produced by immunization of BALB/c mice with promastigote membranes obtained by N_2 cavitation and ultracentrifugation. From several hybridomas produced, three clones showed to be highly specific to *L. (V.) braziliensis* promastigotes. These MoAbs were named SST-2 (IgG1), SST-3 (IgG3) and SST-4 (IgG1). By Western blotting it was shown that: i) MoAb SST-2 reacts with two components (about 22 and 26 kDa), ii) MoAb SST-3 recognizes a glycoprotein (about 160 kDa), and iii) MoAb SST-4 recognizes a component about 98kDa. The antigen reactive with MoAb SST-3 showed to be glycoprotein present the surface of parasite flagella. The SST-3 reactivity was abolished after treatment of the nitrocellulose with sodium m-periodate, indicating that the epitope recognized by SST-3 is present in the carbohydrate moiety of this glycoprotein. These three MoAbs, when analyzed by immunofluorescence, solid-phase radioimmunoassay and Western blotting, did not show any cross-reactity with *L. (L.) amazonensis*, *L. (L.) major*, *L. (L.) chagasi* promastigotes as well as with *Trypanosoma cruzi* epimastigotes. By indirect immunofluorescence MoAbs SST-2 and SST-3 showed to be reactive with other *L. (V.) braziliensis* serodemes, but not with *L. (V.) panamensis*, *L. (V.) guyanensis*, *L. (V.) naiffi* and *L. (V.) lainsoni* promastigotes. These results clearly indicate that MoAbs SST-2, SST-3 and SST-4 recognize specific epitopes of *L. (V.) braziliensis*.

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BC-76
MOLECULAR MARKERS TO IDENTIFY SPECIES OF THE *LEISHMANIA (VIANNIA)* SUBGENUS

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We showed that the coding region of the b-tubulin gene has sufficient variation to accurately discriminate between the *Leishmania* parasites at the subgenus level. Also, we identified by RFLP analysis a diagnostic nuclear DNA sequence of the *Leishmania* genus, b500, which have a high specificity to the species of the *Viannia* subgenus. In this work, we used the same analysis to genotypically characterize different *Leishmania* isolates from Antoquia, Colombia and from Macapo, a village in the Carabobo State, on northwestern region of Venezuela. In a first approach using a restriction enzyme analysis, the results showed that the *panamensis* and *braziliensis* species coexist in different places of the Antoquia region, whereas only *braziliensis* was identified in Macapo. In mixed cultures a RFLP analysis of the coding region of the b-tubulin gene showed sufficient variability to discriminate between both species, *panamensis* and *braziliensis*. In a second approach, a PCR method that amplifies a subgenus-specific sequence present in the b-tubulin gene region, b500, was used for the identification of *L. (Viannia)* on biopsies from patients with CL. Our results confirm the specificity of the b500 sequence and the presence of *braziliensis* species in Macapo. Also, we conclude that a polymorphic Pst I site present in the coding region of the b-tubulin gene is specific to the *panamensis* species.

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BC-77
MODULATION OF ECTO-ATPASE ACTIVITY IN *LEISHMANIA TROPICA* BY SULFATED POLYSACCHARIDES

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Leishmania are dimorphic intracellular protozoan parasites. The flagellated promastigote form is delivered to the mammalian host from an infected sandfly. Promastigote binds to specific receptors on macrophages and enter cells by receptor-mediated endocytosis. It has been described a heparin-binding protein present on the surface of *L. donovani* (Mukhopadhyay et al. *Biochem J 264*: 517-522) and that heparin enhances the interaction of infective *L. donovani* promastigote with macrophage (Butcher et al. 1992 *J immunol 148:* 2879-2886). The presence of an ecto-ATPase (EC 3.6.1.3) has been shown in a wide range of human and animal tissues. We have previously characterized a Mg-dependent ecto-ATPase in *L. tropica* (Meyer-Fernandes et al. 1997 *Arch Biochem Biophys 341*: 40-46).

The physiological role of this enzyme is still unknown but its involvement in cellular adhesion has been postulated. In this study we investigated the effects of sulfated polysaccharides on the ecto-ATPase activity of intact cells of *L. tropica*. We observed that increasing concentrations of dextran sulfate stimulated progressively the ecto-ATPase activity, and this stimulation depends on the pH of the medium and the size of the molecule of dextran sulfate. At pH 7.5, the stimulation was over than 50%, whereas that at pH 8.5 the stimulation was only 25%. When the cells were incubated at pH 7.5 in the presence of dextran sulfate *Mr* 8,000, 40,000 and 500,000 the stimulation of ecto-ATPase activity was 11%, 23% and 63% respectively. This stimulation was not observed when dextran desulfated *Mr* 40,000 and other sulfated polysaccharides such as condroitin sulfate and heparin were tested. The stimulation by dextran sulfate 500,000 on the ecto-ATPase activity was abolished by the increase of the ionic strength of the medium. This activation was also abolished by spermidine and to a lesser extent by putrescine, two polyamine present in *Leishmania*.

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BC-78
METABOLIC AND VIRULENCE CORRELATIONS BETWEEN TWO *LEISHMANIA BRAZILIENSIS* M2903 ISOLATES: PUTATIVE ROLE FOR THE SMALL CHROMOSOME

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The leishmania genome varies from 10^7 to 10^8 bp per haploid genome and its molecular karyotype displays 16 to 24 chromosome within a range from 150kb to 7Mb; a great length polymorphism is noted between species and strains as well as amplification phenomena, with or without drug pressure; these DNA amplifications can be in a circular or linear form. One of them is present in the *Leishmania braziliensis* M2903 strain as a linear 245kb chromosome with unknown function. *Leishmania* are auxotrophic for biopterin and a gene for a biopterin transport protein was recently localized in this small chromosome. We cultivated two M2903 isolates, with (566) and without (2903) the 245kb DNA amplification in M199 medium, and found that the former is very slow growing in relation to the second. When both were grown in the presence of increasing concentrations of biopterin (0 to 640ng/ml) there was no obvious growth difference in isolate 2903, while 566 showed a growth stimulus at low concentrations of biopterin (10 to 80 ng/ml), and concentrations above 80ng/ml were inhibitory. In order to investigate differences in infectivity between these two isolates, mouse macrophages were infected *in vitro*. Isolate 566 containing the small chromosome was shown to be much more infective than isolate 2903 without the small chromosome, indicating a role in infectivity for the small chromosome. We are presently investigating differences in infectivity in hamsters.

BC-79
LEISHPORIN, A CYTOLYSIN FROM *LEISHMANIA AMAZONENSIS*, IS ACTIVATED BY DISSOCIATION OR PROTEOLYSIS OF AN OLIGOPEPTIDE INHIBITOR

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Previous studies from our laboratory have shown that extracts of *Leishmania amazonensis* promastigotes lyses erythrocytes and nucleated cells by a membrane-associated pore-forming protein, named leishporin. We have shown that leishporin must be activated by cytosolic serine-protease(s) from the parasite or by exogenous proteases to express its full cytolytic activity. Two hypothesis could be drawn from our experiments: 1) that active leishporin is produced by limited proteolysis of an inactive precursor and 2) that an inhibitory molecule non-covalently bound to the cytolysin is proteolytically degraded releasing the active cytolysin. In the present work, we show evidence that points to the second hypothesis. We have observed that membrane extracts (m-ext) of *L. amazonensis* promastigotes incubated with increasing concentrations (0, 125, 250, 500 mM) of dissociating agents (urea, guanidinum chloride (Gua-HCl) and potassium chloride) have its hemolytic activity increased, in a dose-dependent fashion. The most efficient dissociating agent was Gua-HCl (500 mM) that produced a 5-fold increase in leishporin activity. These results suggest that the activation of leishporin is due to the dissociation of an inhibitory molecule. When the 500 mM Gua-HCl-treated m-ext is submitted to a filtration through a 10 kDa-mesh membrane, the active leishporin is recovered in the fraction containing the molecules larger than 10 kDa. The fraction containing the molecules smaller than 10 kDa, when concentrated in a 3 kDa-mesh filter, is able to inhibit leishporin cytolytic activity. We have also shown that leishporin can be activated by incubation of m-ext for 24 hr at 37ºC with a novel cytosolic 101 kDa serine oligopeptidase purified from promastigotes of *L. amazonensis*. Taken together our results indicate that leishporin has a non-covalently bound oligopeptide inhibitor with molecular mass ranging between 3 and 10 kDa and is activated by its dissociation or proteolysis.

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BC-80
LEISHMANIA COLOMBIENSIS AND *LEISHMANIA EQUATORENSIS*: PECULIAR *VIANNIA* SPECIES

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The taxonomic criterion used to classify a *Leishmania* species as *Viannia* is the perypilarian pattern of development, with a phase in the hindgut of the sandfly. The use of a sole marker to discriminate this group of parasites can lead to misclassifications. The mini-exon gene non-transcribed spacer has been used as a molecular tool to distinguish different *Leishmania* species. For example, although *L. lainsoni* is taxonomicaly identified as a *Viannia* species, the mini-exon gene non-transcribed spacer revealed that it comprises a very divergent species inside the subgenus *Viannia*, behaving as an independent complex. Recently, we have observed that the variable region of the minicircle molecules of both *L. colombiensis* and *L. equatorensis* are different in length when compared with all the other *Leishmania* species. In order to investigate this discrepancy with another molecular tool, we PCR amplified the mini-exon gene of both *Leishmania* species. The amplified products corresponded to a 400 bp repeat, different from all the other *Viannia* species, which are 250 bp or even from *L. lainsoni* that is 300 bp. The PCR product from *L. colombiensis* was cloned and sequenced. The primary DNA sequenced showed a 89,5% of similarity with *E. schaudinni* and diverged from all the described *Leishmania* mini-exon genes. These results, in association with MLEE, minicircle conserved region sequences, ITS of the rRNA gene analysis and sialidase activity demonstrated that *L. colombiensis* and *L. equatorensis* are very related to *E. schaudinni* and may represent an eventual evolutionary link between the genus *Leishmania* and *Endotrypanum*.

BC-81
LEISHMANIA AMAZONENSIS, L. MAJOR AND *L. MEXICANA* RDNA PROMOTER MINIMAL FUNCTIONAL DOMAINS PRESENT A HIGH LEVEL OF SEQUENCE SIMILARITY

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The rDNA promoter region of *Leishmania amazonensis* has been structurally and functionally characterized (Uliana et al. 1996 *Mol Biochem Parasitol 76*: 245). Unexpectedly, *L. major* and *L. mexicana* transfected with the *L. amazonensis* promoter showed higher levels of CAT expression than the observed in experiments with the homologous system (Stempliuk et al. 1997 *Mem Inst Oswaldo Cruz 92*: 176). To determine the reasons for the differential levels of expression, we characterized the regions mapped as being the promoter minimal functional domains in *L. amazonensis* and in the other species. Two regions, encompassing the putatives UBF plus SL1 binding sites (nucleotides –196 to + 170) and the SL1 binding site alone (–74 to 170) of the *L. amazonensis, L. major* and *L. mexicana* promoter sequences were obtained by PCR, cloned and sequenced. These sequence showed a high degree of identity. Constructs bearing the promoter regions derived from the three different *Leishmania* species, upstream to the CAT reporter gene were used for functional studies on the cross-species activity of these promoters. Transient transfection experiments showed that the region –74 to 170 contains a functional element, although the levels of expression for this sequence were lower than the one observed for original construct pLaD14ASCAT. This observation might be explained by the absence, in that construct, of a fragment of the ETS, in the 3'region, that contains a polypyrimidine track. Other constructs are being made to test the functional role of those domains and their cross-species activity.

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BC-82
LEISHMANIA AMAZONENSIS IN TAXIDERMIZED RODENTS

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Starting in the forties, the National Service of Rural Endemic collected 60,000 rodents in attempt to seek out plague reservoirs. These animals were examined, taxidermized, and kept in archives at the National Historical Museum of the Federal University of Rio de Janeiro (UFRJ). Since many of these animals were collected from areas where leishmaniasis was also present, we decided to investigate if these rodents were parasitized by *Leishmania* through the polymerase chain reaction (PCR). We examined animals from two well-known *Leishmania* endemic

areas: 20 from Baturité, Ceará and 19 from Ilha Grande, Rio de Janeiro. Three skin fragments were collected from each animal. Genomic DNA was isolated by using QIAmp tissue kit (QUIAGEN, California, USA), ethanol precipited and resuspended in TE. Oligonucleotides that amplify the conserved region of minicircle kDNA were used in a hot start PCR. The amplified products were analysed by agarose gel electrophoresis, and dot-blots were hybridized with a preamplified product of *L. panamensis* or *L. amazonensis* as probes. Our results show the presence of a 120bp band in two animals, both from Baturité: one *Oryzomys eliurus* and one *Trichomys apereoides*. These animals were captured in 1953. Hybridization showed that these positive PCR products were *L. amazonensis*. These results raises the possibility to reconstruct the history of leishmaniasis through retrospective studies by using molecular approaches.

BC-83
ISOLATION OF SPECIFIC GLYCOLIPID ANTIGENS OF *LEISHMANIA (VIANNIA) BRAZILIENSIS*

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In order to identify possible glycolipid antigens of *Leishmania (Viannia) braziliensis*, monoclonal antibodies (MoAbs) were produced. BALB/c mice were immunized with promastigote membranes obtained by N_2 cavitation and ultracentrifugation. A hybridoma line was established, and termed SST-1. The monoclonal antibody (IgG3) secreted by SST-1 showed to be directed to the glycolipid fraction of *L. (Viannia) braziliensis* promastigotes. Glycolipids of *L. (V) braziliensis* promastigotes were extracted with mixtures of chloroform/methanol and isopropyl alcohol/hexane/water. The glycolipids were purified by chromatography on Octyl-Sepharose, Silica-Gel 60 and C18 reverse-phase columns and by HPLC using Iatrobeads columns. Reactivity of the MoAb SST-1 with the glycolipids was analyzed by HPTLC immunostaining and solid-phase radioimmunoassay. The glycolipids recognized by SST-1 were present in all *L. (V.) braziliensis* serodemes analyzed. The SST-1 reactivity was abolished after treatment of these glycolipids with sodium m-periodate, indicating that the epitope recognized by SST-1 is present in their carbohydrate moiety. By indirect immunofluorescence and radioimmunoassay no cross- reactivity of SST-1 was observed with promastigote forms of *L. (Leishmania) amazonensis*, *L. (Leishmania) major*, *L. (Leishmania) chagasi*. The structure of *L. (Viannia) braziliensis* glycolipid antigens are under investigation.

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BC-84
INVOLVEMENT OF *LEISHMANIA AMAZONENSIS* PROMASTIGOTES SURFACE PROTEINS IN THE RESISTANCE OF PARASITES TO AUTOLYSIS

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We have recently described in *Leishmania amazonensis* extracts a pore-forming protein, named leishporin, that lyses erythrocytes and nucleated cells including its host cell, the macrophage. In the present work we report that leishporin is secreted by promastigotes. However, parasites are not lysed by its own cytolysin. Parasites were incubated for different periods of time (30 min to 5 hr) at 24ºC and pH 7.2 and the hemolytic activity of the supernatant was determined. As early as 30 min, the supernatant presents hemolytic activity, indicating that promastigotes secrete leishporin in its active form. However, parasites remain intact and viable in contact with its own cytolysin, even after 5 hours of incubation, as revealed by the vital dye Erythrosin B. To investigate the resistance of parasites to autolysis by leishporin, viable promastigotes were incubated with a cytolytic promastigotes extract (p-ext) at 37ºC for 30 min at pH 5.5, the optimal conditions for leishporin full activity. Parasites were stained with Erythrosin B and dead and alive parasites were counted. We observed that parasites are resistant to lysis by p-ext when compared to erythrocytes and nucleated mammalian cells. Parasite resistance increases during its growth *in vitro*, reaching a peak at late logarithmic phase or early stationary phase. To determine whether parasite surface proteins are involved in resistance to lysis by leishporin, viable promastigotes were incubated with proteases (proteinase K, trypsin and chymotrypsin) in different concentrations (6.25, 12.5, 25 and 50 µg/ml) for 2 hr at 34ºC. Parasites were then washed, incubated with p-ext for 30 min at 37ºC and assayed for viability. We found that proteinase K and trypsin caused a dose-dependent reduction of parasites resistance to its own cytolysin. Chymotrypsin, on the other hand, had no effect on parasite's sensitivity to lysis. These data indicate that trypsin-sensitive and chymotrypsin-resistant promastigotes surface proteins are involved in the resistance of the parasite to autolysis.

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BC-85
IDENTIFICATION OF INDIVIDUAL COMPONENTS OF AN IMMUNOGENIC PROTEIN FRACTION FROM *LEISHMANIA BRAZILIENSIS BRAZILIENSIS*

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We propose to characterize the role that the major surface antigen of *Leishmania*, the protein called GP 63, and the 83 kDa heat-shock protein (HSP), might have as components of an immunogenic protein fraction from *L. b. braziliensis* shown previously to induce a protective immune response against leishmaniasis. This fraction was obtained from total parasite extract through fractionation in SDS-PAGE and elution of proteins within the molecular weight range of 67-94 kDa. Degenerate primers were designed for the amplification by PCR of fragments coding relevant parts of the genes for both proteins. The primers were selected based upon published sequences for the *L. b. braziliensis* HSP 83 gene and the GP 63 genes from different species of *Leishmania*. Amplification was followed by cloning the resulting fragments in the pTZ18R cloning vector and subsequently subcloning in the *E. coli* expression vector pGEX2T (both plasmids from Pharmacia), allowing the successful expression of the fragments as recombinant fusion proteins in frame with Glutathione S-transferase. Three different fragments (0.5, 0.7 and 1.1 kb) from the GP 63 gene were amplified, subcloned and expressed as fusion proteins. Partial sequencing of two different clones obtained from the smaller fragment confirmed their identity as GP 63 and also showed that fragments from more than one gene were amplified (several copies of the GP 63 gene have been described from *Leishmania*). The HSP 83 gene was amplified and expressed as two fragments (1 and 2 kb) consisting of the N-terminal half of the protein and the entire coding region. Total *E. coli* extracts obtained from clones producing the different proteins were then tested by Western Blot with rabbit polyclonal serum directed against the 67-94 kDa protein fraction from *L. b. braziliensis*. The serum specifically recognized all the different fusion proteins from both HSP 83 and GP 63 origin indicating that the two proteins are immunologically active components of the fraction. The same serum was then immunoadsorbed with the *E. coli* extracts prior to use in Western Blot with total *L. b. braziliensis* protein. Preliminary results show no significant difference seen in the pattern of proteins recognized by the different immunoadsorbed sera, as compared to control non-treated serum, suggesting that although both HSP 83 and GP 63 are present in the fraction and are capable of inducing a immune response, several other proteins remain to be identified which are probably involved in the induction of protective immunity by the 67 to 94 kDa protein fraction.

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BC-86
IDENTIFICATION AND PARTIAL CHARACTERIZATION OF A NOVEL MEMBER OF THE HSP70 FAMILY IN THE PROTOZOA *LEISHMANIA (LEISHMANIA) CHAGASI*

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Both visceral and tegumentary leishmaniasis are endemic diseases in the northeastern region of Brazil. In spite of the high sequence conservation of the heat shock proteins, members of the Hsp70 kDa family are often found as a dominant antigen in many human infections caused by invading microbes, including leishmaniasis. We used a rabbit polyclonal serum, raised towards an immunogenic fraction of *L. (Viannia) braziliensis* (LbbF2), to perform an immunoscreening of a cDNA library of *L.(L.) chagasi*. The LbbF2 fraction comprises of a range of 67 to 94 kDa protein bands in SDS-PAGE of *L.(V.) braziliensis* promastigote lysate and has tested vaccine potential capabilities against human tegumentary leishmaniasis. As a result of several library screenings with the heterologous anti-LbbF2 serum, we isolated about 8 strongly reactive clones. Two of these clones with inserts of approximately 1.2 and 2.2 Kb were partially sequenced and most detailed studied after *in vivo* pBluescript excision from the original LambdaZAPII vector. Through comparative sequence analysis, the clones proved to be related to the mithocondrial Hsp70 proteins of *Leishmania*. *E. coli* XL1-BLUE was transformed with the pBluescript containing the inserts of interest and expression of the fusion proteins was noted in SDS-PAGE after IPTG induction. Immunoreaction of the fusion proteins with the LbbF2 anti-serum was again confirmed by Western-Blot. When the LbbF2 anti-serum was previously immunoadsorbed with the recombinant bacteria and used in immunoblots of *L.(L) chagasi* lysates, a band of approximately 70 kDa disappeared, thus probably indicating the native protein. In addition, through the same methodology, but instead using *L.(V.) braziliensis* total lysates immunoblots, we also observed that a band of approximately 70 kDa disappeared, suggesting that this or a closely related protein could be a component of the LbbF2 fraction.

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BC-87
GROWING PROMASTIGOTES OF *LEISHMANIA BRAZILIENSIS IN VITRO:* THE EFFECT OF HUMAN URINE

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Leishmania braziliensis promastigotes are difficult to culture *in vitro*. Howard et al., in 1991, demonstrated that human urine improves the growth of *Leishmania in vitro*, stimulating cell division and also facilitating the primary culture of cells from infected animal tissue. The objective of this study is to identify and characterize the factor(s) which is (are) responsible for this phenomenum. Firstly, we have characterized the growth of a strain of *L. braziliensis*, isolate # 2904 (kindly provided by Dr JJ Shaw), in M199 with different supplements. The parasite growth was not sustained in M199/10% FCS, with an inoculum of 1 x 10^5 cells/ml for 7 days. The same inoculum in M199 plus 2% human urine reached 5x10^6 cells/ml after 7 days of culture. M199 supplemented with 10% FCS plus 2% human urine allowed cells to grow to1 x 10^7 cells/ml. In order identify the substance responsible for such effect we fractionated human urine components with Centricon (Amicon, Inc) of two different molecular mass cutoffs: 30 kDa and 10 kDa. When to the M199, supplemented with 30-10 kDa urine fraction, was added 10% FCS the *L. braziliensis* cells reached 1 x 10^7 cells/ml after 7 days of culture, but it has not shown any growth when cultured in the same media without FCS. On the other hand, when the M 199 media was supplemented with the <10 kDa urine fraction whether or not in the presence of 10% FCS the *L. braziliensis* reached a number of 5x10^6 cells/ml after the same 7 days of culture. Finally, M199 supplemented with samples from the >30kDa fraction did not promote growth even in the presence of 10% FCS. One possible explanation for these findings is that there are two putative factors responsible for the stimulation observed in the *L. braziliensis* growth when cultured with human urine. The first one would be FCS-dependent and would permit *L. braziliensis* cultures to reach the best levels of growth in M199 (about 10^7 cells/ml). The second 'factor' would be FCS-independent and would permit some growth, up to 5-6 x10^6 cells/ml. We are currently processing the urine fractions in different ways in order to understand which class of molecules they belong to before choosing appropriate methods for further purification.

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BC-88
GLYCOINOSITOLPHOSPHOLIPIDS (GIPLS) FROM *LEISHMANIA (V.) EQUATORENSIS*: INVESTIGATION ON THE LIPID MOIETY

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Promastigotes grown in BHI-supplemented medium of two *Leishmania (V.) equatorensis* strains isolated from the viscera of a sloth (*Choloepus hoffmanni*) and a squirrel (*Sciurus granatensis*) had GIPLs obtained by hot phenol-water extraction followed purification by solubilization in chloroform-methanol-water (10:10:3). The lipid domain of both GIPLs was chemically analysed and showed to be identical. The long chain bases were identified by gas-liquid chromatography-mass spectrometry (GC-MS) after acid hydrolysis, *N*-acetylation and *O*-trimethylsilylation as being C_{20} and C_{21} -phytosphingosine and the fatty acid composition was determined by GC after methanolysis and hexane extraction being predominantly $C_{16:0}$, $C_{18:0}$ and $C_{24:0}$ fatty acid methyl esters. These results were confirmed by nitrous deamination of intact GIPLs, liberating inositolphospholipids analysed by negative ion fast atom bombardment-mass spectrometry (FAB-MS). The present data showed that the lipid moiety expressed in GIPLs of the two strains of *L. (V.) equatorensis* was similar to that found in GIPLs synthesized by *Endotrypanum* spp.(sloth parasites), whereas, only glycerolipids were found in the GIPLs of *Leishmania* species investigated to date.

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BC-89
GENETIC VARIABILITY OF *LEISHMANIA* IN VENEZUELA

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The leishmaniasis refers to a large spectrum of clinic symptoms caused by protozoa of *Leishmania* genus, with a variety of hosts and transmitted by sand flies. This disease is endemic in tropical areas and can be classified as

visceral leishmaniasis (VL) and cutaneous (CL). In Venezuela, leishmaniasis is endemic throughout the country except the Delta Amacuro, occurring in 12.3/100.000 habitants. The species described as vectors are *L. braziliensis, L. amazonensis, L. guyanensis, L. venezuelensis, L. chagasi*, and *L. colombiensis*. This study aims to characterize the *Leishmania* species isolated in the middle-west area of Venezuela, through enzyme electrophoresis in agarose gels (MLEE), compared to the reference strains suggested by WHO. For this purpose, the strains were characterized using 18 enzymatic systems: 6PGDH, G6PDH, MDH, PGM, ME, PEPD, IDHNAD, IDHNADP, NH, Nhox, GPI, PEP2, PEP3, ACON, , FUM, HK, MPI, ACP. The genetic diversity of these strains is being also evaluated by the RFLP of the internal transcribed of rRNA (IRT) and RAPD. Our data, based on the electrophoretic profiles, indicate the presence of *L. braziliensis, L. venezuelensis, L. guyanensis*, hybrids between *L. braziliensis/ L. guyanensis*, and *L. colombiensis*, being the latter described for the first time in this endemic area of Venezuela. These species were isolated in patients with both, VL and CL. The *L. colombiensis* represent a group with little heterogeneity by both MLEE and IRT, and is a variant of the reference strain isolated in Panama (IGOM/PA/85/E582.34). Furthemore, our results may be interpreted following the new concept that certain species of *Leishmania* show cutaneous and visceral tropism, which may depend on the immunological profile of the host, as the *Leishmania* strains involved in infection.

BC-90
GENETIC RELATIONSHIPS AND EVOLUTION OF NEW WORLD *LEISHMANIA:* SUGGESTION OF EVOLUTIVE LINK WITH *ENDOTRYPANUM*

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Similarities at the morphological, molecular and biological levels exist between many trypanosomatids isolated from sylvatic insects and/or vertebrate reservoir hosts that make the identification of medically important parasites demanding. Molecular data have suggested a close relationship between some *Leishmania* species and *Endotrypanum*, which has an important epidemiological significance and can be helpful to understand the evolution of these parasites. In this study, we have used (a) numerical zymotaxonomy, (b) the variability of the internal transcribed spacers of the rRNA genes and (c) SSU rRNA gene sequences to examine the genetic relationship between *Endotrypanum* and some leishmanial species: *L. (L.) herreri, L. (L.) hertigi, L. (L.) deanei, L. (V.) colombiensis* and *L. (V.) equatorensis*. The phenetic analysis using the isoenzyme data and ITSrRNA show that *L. colombiensis, L. herreri,* and *L. hertigi/L. deanei* are each a heterogeneous group of parasites. The molecular trees obtained revealed high genetic similarity between the six *Leishmania* species and *Endotrypanum*, forming a tight cluster of parasites, with *Endotrypanum* closer to *L. (V.) colombiensis* than to the others. Based on further data from the measurement of sialidase activity, these parasites were also grouped together. In contrast to several other *Leishmania* species and in agreement with the results observed for *Endotrypanum* (Medina-Acosta et al. 1994 *Eur J Biochem 225:* 333), the *Leishmania* closely related to *Endotrypanum* presented positive activity to sialidase, except for one strain of *L. herreri* that was negative. The current taxonomy of *Leishmania* and the evolution of the *Leishmania/Endotrypanum* clade will be discussed.

BC-91
EXPRESSION OF AN ENDOGENOUS INHIBITOR OF CYSTEINYL-PROTEINASES IN *LEISHMANIA*

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Cysteinyl-proteinases are promising target molecules amenable to pharmacological intervention of parasitic development and disease progression, particularly because they participate in fundamental metabolic functions. For example, inhibition of a *Plasmodium* cysteinyl-proteinase with peptide proteinase inhibitors cures murine malaria (Rosenthal et al. 1992 *J Clin Invest 91*:1052-1056). Similarly, *Leishmania* null mutants of the cysteinyl-proteinase genes, engineered by homologous gene replacement, are less virulent *in vivo* (Mottram et al. 1996 *Proc Natl Acad Sci USA 93*: 6008-6013). The leishmanial cysteinyl-proteinases are expressed and differentially regulated during parasite development. The exact molecular mechanism(s) governing the pattern of gene expression is (are) not completely understood. However, both transcriptional and translational controls take place. Post-translational regulation by inhibition of enzyme activation has also been suggested. The endogenous inhibition could be mediated by intramolecular interactions established between the pre-pro-peptide region and the active site, either in the precursor or in the mature polypeptide, resulting in physiological regulation of enzyme activity. Proteinaceous inhibitors, known as cystatins, are naturally occurring in plants. An inhibitor, termed *chagasin*, of a papain-like cysteinyl-proteinase was recently characterized in *Trypanosoma cruzi* (Monteiro et al. 1998 XXVII Reunião Anual da SBBq,

MR10-2, p.xiii). In this work, we wished to determine the occurrence of such inhibitors in the trypanosomatids, by carrying quantitative colorimetric enzyme assays. We detected cystatin-like activities in *Leishmania equatoriensis*. A rabbit polyclonal antibody developed against recombinant cowpea cystatin was used to survey for cross-reactive epitopes in this group of parasites. We are exploring the antibody for further characterization of candidate proteins of endogenous inhibitors in *Leishmania*. We are also setting up transfection experiments to express the exogene that codes for a cowpea cystatin, to re-appraise the biological function(s) of cysteinyl-proteinases in *Leishmania*.

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BC-92
EVIDENCE THAT TRYPANOTHIONE REDUCTASE IS AN ESSENTIAL ENZYME IN *LEISHMANIA* TO PROTECTS THE PARASITES AGAINST NITRIC OXIDE

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The trypanothione system of trypanosomatid protozoa is a thiol-redox cycling system that protects the parasite against damage by oxidants and toxic heavy metals. Trypanothione reductase (TR), a flavoprotein oxidoreductase present in trypanosomatids but absent in human cells, is regarded as a potential target for the chemotherapy of several tropical diseases caused by *Trypanosomes* and *Leishmanias.* In view of the pivotal role and uniqueness of TR in the management of oxidative stress, we investigated whether *L. donovani TryA* disruption mutants with less TR activity than wild type cells (Tovar et al. 1988 *Mol Microbiol 29*: 653-660) have the same ability to survive in presence of reactive oxygen and nitrogen species. We analysed the clones LV9.3 (wide-type - aneuploid trisomic), H2 (single *TryA* replacement), HB3 (double replacement), and pTTcTR (as HB3 plus pTTcTR episome). We have shown that: a) the addition of superoxide anion to the parasites had no significant leishmanicidal effect; b) hydrogen peroxide killed *L. donovani* only high concentrations as 1mM; c) the addition of SNAP directly to the parasites resulted in dose-dependent killing of different clones and d) interestingly, the mutant HB3, cells with highly decreased TR catalytic activity, was more sensible to the cytotoxic effect of NO than the others clones. These results indicate that *TryA* gene product is an essential protective cellular component against nitric oxide in *Leishmania.*

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BC-93
DEVELOPMENT OF AN *IN VITRO* TRANSLATION SYSTEM FROM *LEISHMANIA* SP.

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Cell-free protein synthesising systems, in particular the rabbit reticulocyte lysate (RRL), are powerful tools used for both the characterisation of the translation machinery as well as the understanding of post-transcriptional regulation of gene expression in eukaryotic organisms. Nevertheless, commercially available systems are not entirely applicable for studies of specialised tissues or more primitive organisms. *Leishmania* spp. and protozoan parasites belonging to the *Kinetoplastida* order are very primitive eukaryotic cells, responsible for a series of diseases of world-wide impact. By now, very little improvement has been achieved so far in the development of chemotherapy probably because of the lack of in depth knowledge regarding their basic biological processes. We propose to obtain an effective tool to be used in the study of translation and post-transcriptional control of gene expression in *Leishmania*. The development of such competent and reproducible cell-free translation system was possible by optimising protocols already described by others. Using *L. donovani chagasi* (LDC) promastigote forms grown in culture, we managed to test a number of different conditions for extract preparation, lysate fractioning and translation reactions. Protein synthesis was monitored by performing the reactions in the presence of ^{35}S-methionine followed by analysis of the newly synthesised proteins with SDS-PAGE and autoradiography. At the present, our results have demonstrated that: (i) the type of cellular lysis was crucial for translation efficiency, as shown for *T. cruzi*. The lysis was performed by vortexing the cells ($4x10^9$/ml) in the presence of half volume of glass-beads (diameter of 0.5 mm) for pulses of 30 seconds; (ii) incubating the translation reactions at 20^{o}C allows for *de novo* synthesis, shown by experiments using time kinetics of *in vitro* translation and pulse chase experiments for labelling of newly synthesised proteins. These extracts effectively and reproducibly allowed new protein synthesis; (iii) critical differences were found in LDC lysates eluates from different Sephadex G-25 column. It was previously known that lysates submitted to gel filtration showed better translational efficiency. However we have reproducibly obtained from the same LDC lysates submitted to different gel filtration chromatography (bed volumes of 0,6 cm^3

and 1,3 cm^3), two protein profiles consisting of distinct patterns of elution of the lysate proteins (seen by Coomassie Blue staining) as well as elution of the translation activity. Furthermore the two different activities differed as to the pattern of proteins synthesised as well as their resistance to Micrococcal Nuclease treatment of endogenous mRNAs, indicating that the elution of the cellular translational machinery might comigrate with different subpopulations of mRNPs.

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BC-94
DECREASED VIRULENCE OF *LEISHMANIA MAJOR* TRANSFECTANTS CARRYING AN EXTRA MINIEXON ARRAY

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A physical map for *L.major*-LV39 chromosome 2 containing the miniexon array, was used to carry some functional studies. Samaras and Spithill reported the occurrence of chromosome 2 size alterations in virulent and avirulent clonal lines of *Leishmania* (UCLA Symposia on Molec. Cell. Biol., vol42, p. 269-278, 1986). A clone from the map bearing 40 Kb of miniexon repeats, was transfected into an avirulent line of *L.major* to test this hypothesis. It has been already shown (Mem. Inst. Oswaldo Cruz, vol. 92, suppl. 1997, p. 155) that only one transfectant (D2 clone, among four) showed significant increase in virulence *in vivo* when they were injected on susceptible animals (BALB/c). To test whether this effect is related to the miniexon extracopies, transfectants were cured. Cured clones were selected by a dilution assay. *In vivo* experiments have shown that the virulence observed in D2 clone is not related to the increased number of miniexon. Possible explanations to this effect include (i) the levels of drug pressure that we have tested were not sufficient to detect changes in virulence in all clones (ii) the change of virulence observed could be induced by the transfection procedure (as a source of stress) (iii) an undetected recombination had occured in the exogenous molecule into D2 clone. In order to verify these possibilities, we have performed some experiments: (1) transfectants mantained under 16, 40 and 80 µg/ ml of hygromycin B in culture were used to verify whether higher levels of miniexon expression influences the pattern of virulence and our data suggest that the increased expression of miniexon array causes a decrease in virulence. (2) five transfectants carrying the cosmid vector without any insert were infected in BALB/c to test whether the transfection procedure could be the inducer of increased virulence observed for D2 clone. Data do not confirm the hypothesis. (3) a molecular analysis of the molecule recovered from *Leishmania* transfectant clones was carried out and it shows that recombination did not occur.

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BC-95
CYSTEINE PROTEINASES OF *LEISHMANIA*: ROLE OF SIGNAL PEPTIDES IN TARGETING

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Our Laboratory has been characterizing the abundant amastigote-specific cysteine proteinases (LPCYS2) of *Leishmania pifanoi*, a member of the *Leishmania mexicana* complex. We are presently investigating the mechanisms of cellular targeting of these lysosomal enzymes. In mammals the trafficking mechanisms of lysosomal hydrolases involves mannose-6-phosphate receptors. In trypanosomatids, no phosphorylated mannose residues were found. In yeast, specific aminoacids in the propeptide region of carboxypeptidases appear to be involved in sorting to the vacuole. On the other hand, a long C-terminal extension found in most cysteine proteinases of trypansosomatids was hypothesized to be involved in cellular sorting.

We are investigating the role of the pre-propeptide, and of the C-terminal extension in targeting of LPCYS2 to the lysosome, by fusing them to the reporter Green Fluorescent Protein (GFP). The gene fragments coding for these domains were cloned into pXG-GFP+2' and pXG-'GFP+, developed by S. Beverley et al. These constructs were transfected into *L. pifanoi* and *L. major* and analyzed by fluorescence microscopy. The parasites transfected with the constructs containing the pre-pro region of LPCYS2 fused to the N-terminus of GFP, showed bright localized fluorescence, compatible with a lysosomal compartmentalization of GFP. The GFP-C-terminal domain fusion transfectants showed a very bright cytoplasmic fluorescence, as compared to the cells transfected with the control plasmids containing GFP alone. This might be indicating a stabilizing function for this domain. We have fused both the pre-pro and C-terminal domains to GFP, and are presently transfecting these constructs into the parasites.

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BC-96
CLONING AND PARTIAL CHARACTERIZATION OF TWO METACYCLIC-STAGE EXPRESSED GENES IN *LEISHMANIA*

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Within the promastigote life-cycle stage in the insect alimentary tract, *Leishmania* parasites sequentially develop from proliferating non-infective (procyclic) to non-dividing, infective (metacyclic) forms. In an attempt to dissect the signals inducing the metacyclic differentiation step, genes were recently identified that are predominantly or exclusively expressed in metacyclic forms, including the ORFs meta-1 and mat-1 from *L. major.* Here we report on the isolation and partial characterization of the respective ORFs of meta-1 and mat-1 in *L. infantum* and in the New World *Viannia* species *L. braziliensis* and *L. panamensis*. The genes have been sequenced, the genomic organization determined and their expression analyzed on RNA- and protein level. The analysis of the meta-1 amino acid sequence shows a remarkably high degree of conservation of approx. 98% between these three species and approx. 90% homology with respect to the recently published *L. major* sequence. Southern blot studies point to the existence of a single-copy gene in the meta-1 genomic locus. Preliminar RT-PCR studies revealed equal expression of Meta-1 transcripts in promastigote forms of *L. infantum* and *L. panamensis*, but show also significant differences in the length of the 5´UTRs varying according to the species. The sequence analysis of the mat-1 ORF of *L . infantum* shows a homology of 82% with respect to the mat-1 ORF of *L. major*. The genomic locus consists of two gene copies, probably organized in tandem. Mat-1 transcripts in *L. infantum* contain a relatively small non-translated 5´UTR of approx. 160 bp. Both proteins were expressed in *E. coli* as recombinant proteins, subsequently purified by affinity chromatography and tested in western blots and ELISAs for its antigenicity using sera from visceral leishmaniasis patients obtained from an endemic region in Colombia. Both meta-1 and mat-1 are recognized by antibodies present in these sera, thus demonstrating that these metacyclic proteins may act as antigens during natural course of leishmaniasis disease. The recombinant proteins have also been used for production of specific polyclonal antisera in rabbits

BC-97
CLONING AND EXPRESSION OF A CHAPERONIN 60 GENE IN *LEISHMANIA BRAZILIENSIS*

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Leishmania braziliensis is the causative agent of mucocutaneous leishmaniasis in South America. This organism has a complex life cycle in which it undergoes an obligatory heat shock upon transference from the insect host (ambient temperature) to the mammalian host (34ºC). During this temperature shift, heat shock proteins (HSPs) show an increase in their expression, suggesting a role in the survival and adaptation of *L. braziliensis* to the mamalian environment. We have been studying the expression of HSPs in Trypanosomes, and have cloned and characterized the hsp60 gene in *T. cruzi* strain CL14 (Giambiagi-deMarval et al. 1993 *MBP 59*: 25-31). We found that it is arranged in a tandem repeat, with at least three copies. Engman and colaborators showed that strain PBOL contains two clusters with aproximately ten copies of the hsp60 gene each (Sullivan et al. 1994 *MBP 68*: 197-208).

Using our *T.cruzi* hsp60 gene as a probe, we screened an *L. braziliensis* genomic library constructed in flEMBL3 (kindly provided by Dr U. Lopes) and isolated a sequence whose predicted aminoacid sequence showed 75% identity when compared to the *T.cruzi* hsp60.A portion of this coding sequence was used as a probe for northern blot analysis of RNA from *L. braziliensis* treated for 2 hr at 34 and 37ºC. Two RNA species of 6 and 4.5kb were detected, and their intensities showed a decrease of 11% at 34ºC and a decrease of 53% at 37ºC, when compared to the control (29ºC). Cells treated in the same conditions but submitted to SDS-PAGE, showed an increase in the intensity of a 61kDa protein band at 34ºC (46%). At 37ºC this band was 18% more intense than the control (29ºC). These results are compatible with a postranscriptional control mechanism for *L. braziliensis* hsp60.

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BC-98
CLONING AND CHARACTERIZATION OF THE ARGINASE GENE FROM *LEISHMANIA AMAZONENSIS* AND EXPRESSION IN *PICHIA PASTORIS*

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Arginase is an enzyme involved in the urea cycle, responsible for the conversion of arginine to urea and ornithine. Promastigotes of *Leishmania* have been previously shown to exhibit arginase activity (Camargo , 1979). Clones containing *L. amazonensis* arginase gene were sequenced. The putative ORF is 990 nucleotides long and presents a 40% sequence similary with the *Rattus norvergicus* enzyme and 43% with the human hepatic enzyme. Genomic blots of *L. amazonensis* DNA, digested with various restriction enzymes and probed with the fragment *Pst* I/*Sac* I from the arginase locus, indicated an homogeneous organization at genome level with a low copy number. PFGE caryotype of *L. amazonensis*, *L. major* and *L. mexicana* chromosomes indicated two chromosomal bands hybridizing to the arginase gene in *L. amazonensis*, and showed chromosomal polymorphisms amongst the three species analysed. The *L. amazonensis* probe recognizes, in RNA blots, polyadenylated transcripts of aproximately 4.4 Kb for *L. amazonensis* and *L. mexicana* and 5.0 Kb for *L. major*, in low stringency washes (2 X SSC, 1% SDS at 65ºC). Hybridization with the *L. major* transcript was not detected at high stringency washes (0,2 X SSC, 1% SDS at 65ºC), indicating a lower similarity between the arginase sequence of *L. major* and the probe. The primary structure of the predicted aminoacid sequence was used to simulate the tridimensional conformation of *L. amazonensis* arginase enzyme based on the cristalographic structure of *Rattus norvegiccus*' arginase (Kanyo 1996). There are aminoacid substitutions around the active site in the tridimensional conformation indicating the possibility of designing a selective inhibitor to *Leishmania* arginase. The precise parameters, however, will only be obtained by X-ray and Syncrotron analysis of the enzyme crystal. Aiming at obtaining the purified enzyme, a pair of oligonucleotides was designed for PCR amplification of *L. amazonensis* arginase ORF. The PCR product was cloned into pMOS-blue Blunt (Amersham) vector and the sequence was verified. This clone was then digested with *Eco*RI and cloned into pPIC-9 vector for expression in *Pichia pastoris*. The expression of the arginase gene in *P. pastoris* will open the possibility of obtaining a suficient quantity of pure enzyme to perform the cristalization of the protein and related structural studies.

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BC-99
CLONING AND CHARACTERIZATION OF *LEISHMANIA TARENTOLAE* ADENINE PHOSPHORIBOSYLTRANSFERASE GENE

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Phosphoribosyltransferases (PRTases) are important enzymes involved in the recycling of purine nucleotides. The mammalian cells can synthesize the purinenucleotides de novo and are only dependent on the salvage pathway during phases of rapid cell growth. Parasitic protozoa of the order *Kinetoplastida* however are unable to synthesize purines de novo and rely on the salvage pathway for the recycling of ribonucleotides. Kinetoplastid protozoa from the genus, *Leishmania*, possess three enzymes involved in the salvage pathway, adenine PRTase (APRT) (EC 2.4.2.7), hypoxanthine-guanine PRTase (HGPRT) (EC 2.4.2.8) and xanthine PRTase (XPRT) (EC 2.4.2.22). APRT is responsible for catalyzing the conversion of adenine and a-D-5-phosphoribosyl-1-pyrophosphate (PRPP) into adenosine-5-monophosphate (AMP) and pyrophosphate (PPi) by the anomeric inversion of the ribofuranose ring. This difference in purine nucleotide metabolism between the mammalian host and protozoan parasites has stimulated considerable interest in the salvage pathway as a target for chemotherapy. The *aprt* gene was cloned from a *Leishmania tarentolae* genomic library and the sequence determined. The *L. tarentolae aprt* gene contains a 708 nucleotides open reading frame that encodes a 25 kDa protein. The predicted amino acid sequence has 85% identity to the APRT of *L. donovani*. A recombinant protein was expressed in *Escherichia coli*, purified to homogeneity and found to retain enzymatic activity. The steady-state kinetic parameters were determined for the recombinant enzyme and evidence for a sequential mechanism obtained. The *L. tarentolae* APRT is active as a homodimer in solution, unlike the *L. donovani* enzyme, which is active as a monomer. The circular dichroism (CD) spectra of APRT are consistent with proteins containing a-helix fractions. Initial crystallization trials for the screening of conditions have been performed on the recombinant APRT.

BC-100
CLONING AND CHARACTERIZATION OF A HIGHLY ANTIGENIC PROTEIN FROM *LEISHMANIA (VIANNIA) PANAMENSIS*

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Recent attempts to develop molecularly defined vaccines against Leishmaniasis based on major proteins of the parasite surface have led to the discovery of the Kinetoplastid Membrane Protein-11 (KMP-11) in *Leishmania*

donovani. In the present work we report the molecular and antigenic characterization of this protein in the New World *Leishmania* species, *Leishmania (Viannia) panamensis*. By using two oligonucleotide primers we amplified the entire KMP-11 coding region of American Tegumentary Leishmaniasis causing species, including *L. panamensis*. *L. braziliensis* and *L. guyanensis*. The *Leishmania panamensis* PCR product was cloned, sequenced and the putative aminoacid sequence determined. A remarkable high degree of sequence homology was found when compared with the *Leishmania donovani* and *Leishmania infantum* counterparts (97% and 96%, respectively). By southern blot analysis it was determined that the KMP-11 locus is conformed by three copies of the gene separated by different sized intergenic regions. After subcloning in a bacterial expression vector, the recombinant KMP-11 production was induced and purified by affinity cromatography on Ni-Nta resin. Sera from patients suffering from Leishmaniasis (including cutaneous, mucocutaneous and visceral) were analyzed in order to detect specific IgG anti-KMP-11 antibodies. The immunoblots showed reactivity in 80%, 77% and 100% of patients respectively. When sera from asymptomatic *Leishmania*-infected individuals were tested, 86% showed a weak reactivity against recombinant KMP-11. In a similar assay, it could be shown that 95% of patients suffering from Chagas disease produce anti-KMP-11 antibodies. These results demonstrate that the KMP-11 protein is a strong immunogen during *Leishmania* and *Trypanosoma* cruzi infections in humans. We propose KMP-11 as serologic marker for Leishmaniasis and Chagas disease. Our current efforts focus on the T-cell immunogenicity of KMP-11 in humans in order to determine its potential as a vaccine candidate.

BC-101
CHARACTERIZATION OF THE INTEGRALS MEMBRANE PROTEINS IN THE GLYCOSOME OF *LEISHMANIA MEXICANA*

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In kinetoplastides, including leishmania species, the enzymes which convert glucose in 3-phosphoglycerate (or 1,3 diphosphoglicerate) are located in a peroxisome-like organelle, called glycosome. The glycosomal membrane is the natural interface between the organelle matrix and the cytosol. In the present communication we describe an analysis of the proteins of the glycosomal membrane of *Leishmania mexicana* grown *in vitro*. Promastigotes were harvested at exponential phase, grinded with silicon carbide and submitted to several centrifugations. Glycosomes were finally purified by isopicnic ultracentrifugations. Generally glycosomes presented a latency of 80% for hexokinase and glucose phosphate isomerase. Membrane glycosome proteins were separated by Triton X-114 treatment a 0°C and centrifugated at its "cloud"point temperature (30°C). Acquoses phase contained hydrosoluble proteins while the detergent rich phase contained integral membrane proteins. The proteins were observed on SDS-PAGE (SDS-polyacrylamide gel electrophoresis) after treating. In order to access the orientation in the membrane of the integral proteins, a labelling by 3-sulfo-N-succinimide Biotine was performed on whole glycosomes previously to Triton-X114 treatment. Labelled proteins were transferred to nitro-cellulose and stained in the presence of streptavidin-peroxidase conjugate. Six integral polypeptides were found in the detergent rich fraction and of them presented cytosolic domain. Their molecular weight were: 51, 49, 23, 22, 16 and 14 kDa. A polyclonal antibody obtained against *Trypanosoma cruzi* glycosomal integral membrane proteins was able to recognise the polypeptides of 51 and 49 kDa of *Leishmania mexicana*, indicating very little homology between the glycosome membrane proteins of the two parasites.

BC-102
CHARACTERIZATION OF THE BINDING MECHANISM OF LEISHPORIN TO THE CELL MEMBRANE

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Leishmania amazonensis express a pore-forming protein, named leishporin, that lyses erythrocytes and nucleated cells, including the macrophage, its host cell. We have previously shown that pore formation involves two distinct stages: 1) the binding and 2) the insertion and/or oligomerization with subsequent cell breakage. To characterize the binding mechanism of leishporin to the cell membrane, we have examined the role of cell surface molecules, using human erythrocytes (HuE) as targets. To investigate the participation of membrane proteins, we have incubated HuE with pronase, proteinase K or trypsin (0, 12.5, 25, 50, 100, 200 and 400 mg/ml) for 1 h at 37ºC. Cells were then incubated with a cytolytic promastigote extract and their sensitivity to lysis was determined. We have found that none of these treatments affected HuE sensitivity to lysis, indicating an insignificant role of HuE surface

proteins in the binding of leishporin to the cell membrane. Aiming to determine the role of membrane lipids in the cytolysin binding mechanism, the promastigote extract was incubated with multilamelar liposomes (20 mM cholesterol, 25 mM dipalmithoilphosphatidylcholine) for 30 min at room temperature. Liposomes were then removed by microcentrifugation at 14.000 rpm for 30 sec and the hemolytic activity of the supernatant was determined. We verified that the liposomes totally removed the hemolytic activity of the parasite extract, indicating the binding of leishporin to one or to both lipids. The kinetics of liposome biding to leishporin was studied by incubation of parasite extract with the liposomes for different periods of time. We verified that liposome-leishporin binding must be immediate since the hemolytic activity is removed at the quickest contact possible of the parasite extract with the liposomes. Our results indicate that lipids, but not proteins, are involved in the binding of leishporin to HuE.

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BC-103
CHARACTERIZATION OF THE ARGINASE GENE IN TRYPANOSOMATIDS

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The activity of arginase, an enzyme of the urea cycle which catalyses the conversion of arginine to urea and ornithine, varies according to a genus pattern within organisms of the Trypanosomatidae family and is a useful character for identification of these organims. In spite of the lack of arginase activity in part of the family, a probe derived from the *Leishmania (L.) amazonensis* arginase gene hybridized to genomic DNA of representatives of all eight genera in the family, indicating the presence of the nucleotide sequence of the enzyme. A PCR fragment was amplified from *Trypanosoma cruzi* genomic DNA with degenerated oligonucleotides derived from the central region of the *L. (L.) amazonesis* arginase gene.

The sequence obtained from this *T. cruzi* fragment showed 60% identity when compared to the same region of *L.(L.) amazonesis* arginase sequence. Extending the characterization of this gene in the family, genomic DNA of *Crithidia fasciculata* was also PCR amplified with the same oligonucleotides and the product will be cloned into pMOS or pUC19 vectors for sequencing determination. If we consider that *L.(L.) amazonesis* and *C. fasciculata* present enzyme activity and *T. cruzi* does not, the comparison of the three sequences will provide clues for determining the natural implications in the evolution of this sequence within the genome of the trypanosomatid family.

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BC-104
CHARACTERIZATION OF AN UNKNOWN GENE IN *LEISHMANIA MAJOR* FRIEDLIN

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In the last few years lots of new genes are being discovered with the emerging of new genome projects such as the "*Leishmania* genome project" which generated a huge amount of information through sequencing. A 70% of these sequences do not have homology to any other known protein, which makes them good vaccine candidates as well as chemotherapeutic targets. One of these genes (Lmk430) was chosen taking into consideration its distribution along the genome. It is allocated at the chromosomal band of 530kb and the physical map of a region of 40kb flanking the gene, shows it is a single copy. Three stop codons are found in the sequence of the EST, a characteristic not usual which was confirmed by sequencing the genomic DNA. The biggest ORF (open reading frame) of Lmk430 has got 246bp and encodes a 942kDa protein. A hydropathy analysis (Kate & Doolittle, 1982) shows the presence of many hydrophobic amino acids. The gene contains motifs for phosphorylation, myristoylation and cell attachment sites. As a tentative to investigate the function of this gene, the ORF with a flanking region of 1.5 kb at both sides of the gene, was cloned into pX63-Neo and the construct obtained has been transfected into *Leishmania major* Friedlin. Recovered transfectants from the solid media will be submitted to high levels of drug pressure to induce over-expression of Lmk430. An evaluation of metacyclogenesis of the parasite was done and control experiments on RNA such as expression of Lmk430 at the different stages of growth of the parasite are under way. To localize intracelularly the product of the expression of the gene Lmk 430 using the reporter protein GFP as a marker, the vector containing the GFP has been transfected into the parasite and an over-expression has been induced using high levels of G418 (1mg/ml). A homogeneous distribution of green fluorescence is observed along the parasite and will be used as a negative control in the localization of the gene product after its fusion with the GFP.

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BC-105
ANALYSING RECOMBINANT COSMIDS FROM DIFFERENT CHROMOSOMAL ENDS OF *LEISHMANIA MAJOR*

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Telomeric and sub-telomeric sequences have been implicated in genome plasticity of parasites and mechanisms of host immune-response evasion. We have previously presented clones bearing the telomere hexameric repeat GGGTTA (Pedrosa et al. 1997 *Mem Inst Oswaldo Cruz 92* suppl. 1: 177), that were rescued from a genomic library of LV39 lineage of *Leishmania major* (Ryan et al. 1993 *Gene 131*:145). We have selected 3 clones (named T, U, and Y) for further analysis. Clone T had been previously associated with chromosome extremities in Bal31 assays (Tosi et al. 1997 *Parasitology 114*: 521) and similar assays confirmed the extreme chromosomal location of clones U and Y in *L. major*. Southern blot analysis of *Leishmania* PFGE-separated chromosomes indicates that clones U, T and Y are originated from specific and different chromosomal bands of 350, 580 and 760 kb, respectively. These results confirmed our previous data suggesting that the selected clones came from different chromosomal ends of the parasite. We will present the complete restriction maps of clones T, U and Y enzymes *Bam*HI, *Bgl*II, *Eco*RI and *Hin*dIII. Restriction mapping and Southern analysis revealed the presence of chromosome-specific fragments, reiterated sequences present in several chromosomes (T-BH 4.5) and, also, sequences repeated internally in the clone Y (Y-BB1.9 and Y-BB 3.0). We are currently undertaking an analysis of the transcriptional activity into these clones. Preliminary data suggests the presence of a multi-copy gene transcribed in clone Y; this region must be further analyzed. Aiming the over-expression of putative genes present in the telomeric clones, we transfected clones T, U and Y in *Leishmania major*. These transfectants are currently kept under high levels of drug pressure and the overexpressed mRNAs will be used as tools for the construction of a transcriptional map.

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BC-106
ANALYSES OF DRUG RESISTANCE PATTERNS AMONG NEW WORLD *LEISHMANIA* SPECIES/STRAINS

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Treatment of leishmaniasis is in general based upon the use of pentavalent antimonial compounds. Response to treatment varies according to either the parasite species involved or the clinical form of the disease. For instance, infections with *L. braziliensis* are usually less responsive to treatment than to those caused by *L. mexicana* (Marsden & Jones 1985 *Leishmaniasis I*: 183-198). Furthermore, treatment is usually more effective at the early stages of the disease. In this study we have tested the in vitro response of several genetically distinct *Leishmania* species/strains to the followed drugs: Itraconazole (ITZ), Terbinafine (TBF), Allopurinol (ALP) and Sodium antimony gluconate (ANTM). Parasites at late log growth phase were incubated in M199 / 10% of FBS in the presence of increasing drug concentration. At the end of the log phase the number of viable cells was determined and the EC50 defined. The parasites tested were classified in a previous study (Cupolillo et al. 1994. *Am J Trop Med Hyg 50:* 296) as: *L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) naiffi, L. (V.) lainsoni, L. (V.) colombiensis, L. (V.) equatorensis, L. (L.) amazonensis*, and *L. (L.) chagasi*. Six strains of *L. (V.) braziliensis* were isolated in Rio de Janeiro (Brazil) from patients presenting different clinical forms. Our results show distinct patterns of resistance to the most of *Leishmania* species/strains for each drug tested. However, the in vitro parasite responses to the drugs could not be correlated with either the *Leishmania* species involved or the level of clinical response of the patients to treatment with related or non-related specific drugs. In addition, the resistance detected in some *L. braziliensis* parasites to ANTM could not be associated with the level of response to the especific treatment. Experiments are being carried out to (a) determine if there is any relationship between drug resistance pattern and genetic profiles, and (b) to further investigate if these parasites can be differentiated based upon isolated loci related to drug resistance of these and other compounds.

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BC-107
SUBTLE MUTAGENESIS BY ENDS-IN RECOMBINATION IN MALARIA PARASITES

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The recent advent of gene targeting techniques in malaria (*Plasmodium*) parasites provides the means for introducing subtle mutations into their genome. Here, we used the TRAP (thrombospodin-related anonymous protein) gene of *P. berghei* as a target to test whether an ends-in strategy, i.e., targeting plasmids of the insertion type, may be suitable for subtle mutagenesis. We analysed the recombinant loci generated by insertion of linear plasmids containing either base-pair substitutions, insertions or deletions in their targeting sequence. We show that plasmid integration occurs via a double-strand gap repair mechanism. Although sequence heterologies located close (less than 450 base pairs) to the initial double-strand break (DSB) were often lost during plasmid integration, mutations located 600 bp from the DSB and farther were frequently maintained in the recombinant loci. The short lengths of gene conversion tracts associated with plasmid integration into TRAP suggest that an ends-in strategy may be widely applicable to modify plasmodial genes and perform structure-function analysis of their important products.

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BC-108
GLYCOSPHINGOLIPIDS IN THE INTRAERYTHROCYTIC STAGES OF *PLASMODIUM FALCIPARUM*

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Although there are many reports on the study of malaria phospholipids, less is known about ceramide-based lipids in *Plasmodium falciparum*. Glycosphingolipids (GSL) are amphipatic glycoconjugates containing an oligosaccharide structure linked to a lipid moiety, the ceramide. The fact that ceramide-based lipids are important for cell growth and development together with the reports on the biosynthesis of N- and O-linked glycoproteins, prompted us to search for the presence of GSL in *P. falciparum*-infected erythrocytes. Cultures of *P. falciparum* (5% hematocrit, 15% parasitemia) were metabolically labeled with [U-^{14}C]-palmitic acid coupled to defatted BSA, during 18 hr. Each stage was purified on a 40/70/80% discontinuous Percoll gradient (15000 x g, 30 min, 25°C). A total lipid extract obtained with chloroform: methanol (1:1) was fractionated by anion-exchange chromatography in DEAE-Sephadex A-25 (acetate form) eluted with chloroform : methanol: water (30:50:8), to recover neutral lipids and zwitterionic phospholipids (fraction A). Acidic lipids were subsequently bulk eluted with chloroform : methanol : 0.8M NaAcO (30:60:8, fraction B). Fraction A was treated with 0.1M NaOH in methanol during 3h at 37°C to saponify phospholipids; after neutralization and concentration on a Sep-Pack C18 cartridge, samples were further fractionated on a silicic acid chromatography column. Free fatty acids were eluted with chloroform and GSL were obtained with chloroform : methanol. Although the radioactivity recovered in the GSL fraction was very low, analysis by thin layer chromatography showed the presence of at least 2 components with mobilities similar to tri- and tetra-hexosylceramides. A labeled spot coincident with a standard of sphingomyelin was also observed. Even though the presence of a parasite sphingomyelin synthase (a marker of Golgi system) has been already determined, this is the first report on the synthesis of GSL in the three intraerythrocytic stages of *P. falciparum*.

BC-109
GENETIC IMMUNIZATION PROTECTS MICE AGAINST LETHAL CHALLENGE WITH BLOOD STAGE PARASITES OF *PLASMODIUM CHABAUDI CHABAUDI* PC7

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Due to their relative simplicity, applicability to various pathogens and, especially, their ability to induce a potent immune response, DNA vaccines seem a very promising new approach to vaccination. Accordingly, we have concentrated our efforts to develop DNA vaccine candidates against the asexual blood stages of malaria in the *P. chabaudi*/mouse model, using two different approaches. i) Firstly, we are seeking parasite blood stage antigens directly involved in protection by Expression Library Immunizations. Thus, cDNA libraries from early trophozoites and from schizonts have been constructed in typical eukaryotic expression vectors and were used in genetic immunization of Balb/c mice. After two boosts, low levels of antibodies against whole P. chabaudi protein extracts were detected by ELISA. The specifity of the humoral immunresponse was confirmed by Immunofluorescence assays and Western blots. ii) The C-terminal region of the Merozoite Surface Protein 1 (MSP1.19) of *Plasmodium* is considered to date the most important subunit vaccine candidate against the asexual blood stages. We have therefore cloned this segment of the gene of *P. chabaudi* (PC7 strain) and *P. vivax* in frame with the HBsAg gene in a vector containing various CpG motifs and driving extracellular localization of the expressed products. After triple immunization of Balb/C mice, relatively high antibody titers (1:12500 in ELISA) were detected in serum samples from mice immunized with this construct as opposed to the titers detected when the same construct without the HBsAg

gene segment was used. Significantly, both approaches have protected mice against lethal challenges, albeit to different extents, depending on the challenge dose and/or the complexity of the injected libraries. Currently, we are testing the reproducibility of these results and analysis of the humoral and cellular immune responses elicited by these two different approaches, will be presented.

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BC-110
EFFECTS OF HYDROXYMETHYLGLUTARYL-COENZYME A REDUCTASE INHIBITORS ON COENZYME Q BIOSYNTHESIS IN *PLASMODIUM FALCIPARUM*

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Coenzyme Q plays an essential role in the mitochondrial respiratory chain, and it acts also as an antioxidant, protecting lipids from lipid peroxidation and free-radical induced damage. The objectives of this work are determine the structure and origin of the isoprenic chain of coenzyme Q in *Plasmodium falciparum*, and as well to test the effect of hydroxymethylglutaryl coenzyme A reductase inhibitors on coenzyme Q synthesis. *P. falciparum* was cultivated in human blood cells according to Trager and Jensen's method and radiolabelled with [1-^{14}C] acetic acid, [1-^{14}C] isopentenyl pyrophosphate, [1-^{14}C] acetyl-coenzyme A, [1(n)-^{3}H] farnesyl pyrophosphate, [2-^{14}C] mevalonic acid and [5-^{3}H] mevalonic acid. Intraerythrocytic stages (young trophozoites, old trophozoites and schizonts) were separated using discontinuous Percoll^{0} gradient, and lyophilised samples from each stage were extracted several times with hexane. The obtained extract was analysed by thin layer chromatography and high performance liquid chromatography. Our results show that *P. falciparum* coenzyme Q's isoprenic chain seems to be synthesised via the mevalonate pathway, and that coenzyme Q_8 is present in all stages of *P. falciparum*. Parasites treated with the hydroxymethylglutaryl-coenzyme A reductase inhibitor mevastatin showed a decreased capability of synthesising coenzyme Q in all intraerythrocytic stages. Our group is testing other inhibitors of the mevalonate pathway, which can represent new options for malaria treatment.

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BC-111
ALLELIC DIVERSITY AND ANTIBODY RECOGNITION OF THE *PLASMODIUM FALCIPARUM* MEROZOITE SURFACE PROTEIN-1 (MSP-1) IN THE BRAZILIAN AMAZON

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The *Plasmodium falciparum merozoite surface protein-1* (MSP-1) locus codes for a major asexual blood-stage polymorphic antigen currently considered as a strong malaria vaccine candidate. Little is known about the patterns of allelic diversity of MSP-1, and the potential impact of sequence diversity on the immune recognition of this antigen, in hypoendemic regions. Here we used the polymerase chain reaction (PCR) to investigate *MSP-1* allelic diversity in 226 *P. falciparum* isolates collected over a period of 12 years across the Brazilian Amazon. No significant differences were found in *MSP-1* allelic frequencies in the variable blocks 2 and 10 when we compared isolates from different areas of the Amazon Basin collected in the same period (1985-6). However, comparisons between isolates collected in the southwestern State of Rondônia at different occasions (1985-89 and 1995-97) showed a decrease in the allelic frequency of block 2 family RO33 and an increase in the allelic frequency of the family K1. These changes may be due to either random genetic drift or immune-mediated frequency-dependent selection. Moreover, we assessed the patterns of antibody recognition of MSP-1-derived conserved (block 3) and variable (blocks 2 and 6-8) recombinant peptides among *P. falciparum*-infected Amazonians with different levels of past malaria exposure (n = 90), and in paired serum samples collected from acute and convalescent malaria patients (n = 20). Results may be summarized as follows: (a) cytophilic antibodies of the IgG subclasses IgG_1 and IgG_3 predominate in antibody responses of most subjects, and the magnitude of IgG_3 responses was positively correlated with the levels of past malaria exposure; (b) antibody responses predominate against variable regions and tend to be allelic-specific; (c) current infection with parasites carrying a particular block 2 allelic variant boosts essentially variant-specific IgG3 antibody responses, which are short-lived. These results are discussed in relation to the possible inclusion of MSP-1-derived peptides in sub-unit malaria vaccines.

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BC-112
CLONING AND EXPRESSION THE GENOMIC LIBRARY OF *TOXOPLASMA GONDII* IN THE MUSCLE OF BALB/C MICE

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Infection with *T. gondii* parasite affect to immunocompromised patients as those with AIDS, neoplastic diseases, transplant organ and congenital diseases. In veterinary medicine, Toxoplasmosis has economic importance due to cattle abortion and neonatal loss. Thus, the development of an effective vaccine against *T. gondii* would be of a great value to both human and veterinary medicine. Nucleic acid vaccine represents a new approach to the control of infectious agent. This novel vaccine are both easy to construct and produce. Recombinant DNA technology is used as immunogens into an eukariotic expression vector. The expression of the plasmid DNA by cells in the inoculated host could generate antibody, cytolytic T cell (CTL) and protective immune responses. The first step in that sense is to achieve the expression of genes of interest in the target tissue. The genomic DNA of *T. gondii* was digested by the restriction enzyme Sau3^{a}, and then ligated into Bam HI site of dephosphorylated pcDNA 3 plasmid (invitrogen) which carried the intermediate early promoter of Cytomegalovirus. In order to check the presence of inserts of *T.gondii* DNA, the transformation with the library were digested with the restriction enzymes EcoRV and BamHI. The construction of the genomic library was obtained by the alkaline lysis method from 21 colonies chose at random among of *E. coli* (HB101) transformants obtained in LB-aga with ampicillin, Three days after the immunization, the animals were sacrificed and tissue section of 4 mm, were cut by cryostat. The samples obtained from these animals (inoculated with genomic library, inoculated with pcDNA 3 plasmid, inoculated *T. gondii* antigens and non-inoculated animal groups) were incubated with a high titer human serum against *T. gondii* extracted antigenic, determined by IFA or with a negative sera pool. The detection of expression in muscle was carried out after three days. In the animals immunized with genomic library plasmid, strong fluorescent staining was observed. The observed signal varies among the different cross-sections of the same animal, being the strongest signal the deepest ones. No significant difference was observed between the animals of this group. In contrast, remaining control groups gave negative results, as expected. Negative results was obtained after incubation with the pool of negative sera in all samples. The level of expression depends on the antigens. The obtained results demonstrates the expression of a genomic library of *T. gondii* in the muscle of immunized of mice.

BC-113
ULTRASTRUCTURAL DETECTION OF ABSENCE OF PHAGOLYSOSOMAL FUSION DURING THE INTERACTION OF *TOXOPLASMA GONDII* WITH HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

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In congenital toxoplasmosis, infection of the cells lining the umbilical cord blood vessels is a major transmission route to the foetus. Therefore, the study of the invasion by *Toxoplasma gondii* of cultured human umbilical vein endothelial cells (HUVEC) is of great interest since these cells are at the interface between the mother and the foetus. *Toxoplasma gondii*, an obligate intracellular parasite, invades and proliferates in HUVEC where it resides in a parasitophorus vacuole. To investigate the process of phagosome-lysosome fusion at ultrastructural level, peroxidase-gold complex was employed as a marker for secondary lysosomes. Endothelial cells obtained from human umbilical cords were labeled with peroxidase-gold complex (10 nm) for 2h at 37°C and then infected with tachyzoites of *T. gondii* (RH strain) for different periods of time (2 and 24hr) at 37°C. The cells were fixed and routinely processed for transmission electron microscopy. After endothelial cell-parasite interaction, the gold particles were observed in cytoplasmic vesicles. Even after 24 hr of interaction no gold particles were observed in any parasitophorus vacuole which indicates absence of phagosome-lysosome fusion during the interaction with *T gondii*.

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BC-114
STUDIES OF THE NON-MITOCHONDRIAL CA^{2+} TRANSPORT SYSTEM OF *PHYTOMONAS SERPENS* PROMASTIGOTES

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In previous work, we have demonstrated that at least two compartments are involved in maintaining Ca^{2+} homeostasis in *Phytomonas serpens* promastigotes: a vanadate sensitive (VS)-Ca^{2+} transport and mitochondria. The response of promastigotes mitochondrial membrane potential to ADP, oligomycin, Ca^{2+} and antimycin A, indicated that the *P. serpens* mitochondria behaved similarly to that from vertebrate and other trypanosomatids regarding the properties of their electrochemical proton gradient and Ca^{2+} transport. In this study, we investigated with more detail the VS-Ca^{2+} transport in digitonin-permeabilized *P. serpens* promastigotes. Ca^{2+} uptake was sustained not only by ATP but also by GTP, however with only 30% of the velocity. High vanadate concentration (1 mM) was needed to get maximal inhibition of the VS- Ca^{2+} transport. The vanadate inhibition presented a lag phase of 2-3 min, which was not observed when promastigotes were preincubated with nigericin. This effect is still not clear and is under study. Nigericin (1mM), FCCP (1mM) or bafilomycin (5mM) had no effect on the VS- Ca^{2+} transport. This suggest the absence of a Ca^{2+} transport mediated by a Ca^{2+}/H^{+} antiport mechanism in the vanadate sensitivity compartment. The vanadate sensitivity Ca^{2+} transport was insensitive to thapsigargin (1mM). Myo-inositol 1,4,5-tris-phosphate (4 mM) was not able to release the Ca^{2+} accumulated by ATP. Some of these effects described suggest that this compartment can be the endoplasmic reticulum (ER). However, it differs from the mammals ER in its insensitivity to IP_3 and thapsigargin. We found no evidence for the existence of a third compartment with the characteristics of the acidocalcisomes described by Vercesi et al. (1994 *Biochem J 304*: 227-233).

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BC-115
STRUCTURAL STUDIES ON GLYCOINOSITOL PHOSPHOLIPIDS OF *TRYPANOSOMA* (*SCHIZOTRYPANUM*) SPP FROM BATS

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Among the four subgenera of *Trypanosoma* recognised as occurring in bats, the *Schizotrypanum* forms are difficult to separate into defined species in view of the general morphological similarity of all the trypanosomes of this subgenus. *Trypanosoma* (*Schizotrypanum*) spp. are parasitic of diverse mammals of the New World, and unless for T. (S.) cruise there is good evidence that bat trypanosomes from this subgenus have a cosmopolitan distribution and are host-restricted to these animals. In this work, we have investigated the structural composition of glycoinositol phospholipids (GIPLs) in epimastigote forms from two bat *Trypanosoma* species that belong to the Schizotrypanum subgenus, T. (S.) *dionisii* and a trypanosomatid isolated from *Phyllostomus hastatus* (Teixeira et al. 1993 *Parasitol Res 79*:497-500). After a two-step purification method employing octyl-Sepharose and Silica-gel chromatography, GIPL-like molecules were detected by negative-ion mode electrospray-mass spectrometry (ES-MS). The phosphatidylinositol (PI) moieties released after HNO2 deamination and NaB3H4 reduction revealed the presence of ceramide made up of sphingosine and alkylacylglycerol-anchored structures containing palmitic acid and stearic acid. By using chemical treatments, sequential exoglycosidase digestion, methylation-linkage analysis and positive- and negative-ion mode ES-MS, the structures of the two major type-1 GIPLs from T. (S.) dioxins were determined. The same Mana1-2(NH2-CH2CH2-HPO4-)Mana1-2Mana1-6Mana1-4(NH2-CH2CH2-HPO3-) HexN-PI core was detected in both structures, and terminal Galf residues substitutions were observed either on the terminal nonreducing Man, as in T. (S.) *cruzi* major GIPLs in strains G and G-645 (Carreira et al. 1996 *Glycoconjugate J 13*:955-966), or in the second a Man residue distal to inositol. The results document the structural heterogeneity of *Trypanosoma* (Schizotrypanum) spp. GIPLs, the most abundant surface component of many trypanosomatids studied so far (Ferguson 1997 *Phil Trans R Soc Lond 352*: 1295-1302), and demonstrate that closely related species in this subgenus might share similar structures.

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BC-116
RANDOM AMPLIFIED POLYMORPHIC DNA FINGERPRINTING (RAPD) ANALYSIS REVEALS THE GENETIC HETEROGENOUS POPULATION STRUCTURE OF *ENDOTRYPANUM* PARASITES

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Parasitic protozoa of the genus *Endotrypanum* (Kinetoplastida: Trypanosomatidae) are a biologically diverse group of microorganisms. Considerable progress in defining the molecular and biological characteristics of these microorganisms has recently been made. Since the description of the genus *Endotrypanum* by Mesnil & Brimont in 1908 the number of strain variants of this parasite has increased, although only two species have been described (*E. schaudinni* and *E. monterogeii*). In previous study reporting population diversity within this genus (Franco et al. 1996 *Parasitology 113*: 39), we have analysed enzyme polymorphism among a group of *Endotrypanum* parasites (17 stocks isolated from sloths in the Amazon Region in Brazil and 6 reference strains). The strains were classified into zymodemes and through numerical analyses the genus was shown to be monophyletic and the 12 zymodemes characterized could be divided into three groups (A, B, C). In parallel study using (a) PFGE and (b) schizodeme (by comparison of kDNA fragment patterns from parasite strains) analyses, the selected *Endotrypanum* zymodemes could also be clustered into distinct groups of parasites of this genus (Franco et al. 1998 *Am J Trop Med Hyg*, in Press). In this study, the heterogenous population of *Endotrypanum* have also been studied at the genotype level by RAPD with selected philogenetically informative primers [M13-40 ("forward" /5'GTTTTCCCAGTCACGAC3'), l15996 (5'CTCCACCATTAGCACCCAAAGC3'), lambda GT11-F (5'GACTCCTGGAGCCCG3') and 3307 (5'AGTGCTACGGT3")] to see wheter this approach could be used to differentiate further among these parasites. The level of genetic variation among parasites was measured by the percentage of shared bands. The genetic variability found among strains representatives of each main isoenzymatic cluster of the genus (including the parasites classified as *E. schaudinni* and a *E. monterogeii*) apparently correlates with our previous reported data. In addition, (a) distinction among the strains classified as zymodeme 01 (EZ01) were detected using primer 3307 and (b) the strains analyzed did not group on the basis of their geographic origin.

BC-117
INTER- AND INTRA-SPECIFIC VARIATION OF *EIMERIA* SPP. OF DOMESTIC FOWL DETECTED BY RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

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Organisms belonging to the genus *Eimeria* are obligatory intracellular parasites that cause a variety of diseases in domestic animals, commonly known as coccidiosis. Seven different *Eimeria* species are involved with a severe intestinal disease in domestic fowl, characterized by diarrhea, loss of weight, listlessness and anemia, leading to important economic losses in poultry production. Identification of the different species is actually made by some tedious and often doubtful techniques, including oocyst shape and size, prepatent period and site of infection. Strain discrimination is more difficult, since intra-specific variations are detected by laborious methods like isoenzyme profiles. Precise identification of *Eimeria* species and strains is required for selection of anticoccidial drugs, diagnosis and epidemiological studies. The present study aimed at discriminate *Eimeria* species and strains isolated in Brazil, using the random amplified polymorphic DNA (RAPD). Since the fact that reference *Eimeria* strains are not available in Brazil, a total of six isolated strains from the three more pathogenic species were employed for an initial survey: four strains of *E. acervulina*, one strain of *E. maxima* and one strain of *E. tenella*. Rapid DNA purification from sporulated oocysts was standardized as well as RAPD reaction parameters. For the initial screening, 38 decamer oligonucleotides (Operon Technologies, Inc.) were tested as primers. The best results were obtained using 20 ng of template, 15 ng of primer, 1 U of Taq DNA Polymerase (Pharmacia), 1,5 mM $MgCl_2$, 250 mM dNTP in a final reaction volume of 20 mL. Following an initial denaturation at 94°C for 5 minutes, amplification was performed in a thermocycler programmed to 40 cycles of 1 min at 94°C, 1 min at 35°C and 1 min and 30 s at 72°C, and a final extension step of 8 min at 72°C. A typical RAPD profile generated 5 to 10 DNA fragments ranging from 300 to 1,600 bp. We found 13 primers that gave multiband profiles that allowed for a high discrimination of the three species analyzed. Six primers resulted in molecular markers associated with intra-specific variation of *E. acervulina*. Further studies employing a higher number of *Eimeria* strains and primers are underway.

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BC-118
ECTO-PHOSPHATASE ACTIVITIES PRESENT ON THE SURFACE OF *ENTAMOEBA HISTOLYTICA*

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Phosphatase activity has been characterized in some members of the Trypanosomatidae family such as *Leishmania*. In *Leishmania* spp, acid phosphatase activity is supposed to be a marker of virulence and it is considered to be one of the mediators of *Leishmania*-macrophage interaction. We have recently characterized ecto-phosphatase activities in another parasitic protozoan of *Trypanosoma* genus (Fernandes et al. 1997 *Z Naturforsch 52c*: 351-358, Furuya et al. 1998 *Mol Biochem Parasitol 92*: 339-348). It has been suggested the involvement of these enzymes in some physiological process, such as nutrition, cell differentiation and in the interaction with its host cells. *Entamoeba histolytica* is a human pathogen with the potential to destroy many tissues of its host. Here we show that live *Entamoeba histolytica* HM1:IMSS strain is able to hydrolyze extracellular *p*-nitrophenylphosphate (*p*-NPP). The parasites were grown in TYI-S-33 medium for 24-48 hours at 37ºC. Cellular viability was assessed, before and after incubations, by eosin method. *p*-NPP was hydrolyzed at a rate of 7.5 nmoles Pi x h^{-1} x 10^5 cells in the presence of 5 mM $MgCl_2$, pH 6.4 at 30ºC and it was reduced 33% (5.0 nmoles Pi x h^{-1} x 10^5 cells) in the absence of divalent metal (basal activity).The Mg-dependent *p*-NPP hydrolysis was strongly inhibited by vanadate, molybdate and sodium fluoride (NaF) but not by levamizole. The Mg-independent basal phosphatase activity was also insensitive to levamizole and less inhibited by vanadate, molybdate and NaF. At the pH range from 6.4 to 8.4, in which the cells were alive, both activities decreased with the increasing of pH.

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BC-119
ECTO-ATP-DIPHOSPHOHYDROLASE ACTIVITIES PRESENT ON THE SURFACE OF *ENTAMOEBA HYSTOLYTICA*

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Entamoeba hystolitica is a human pathogen with potential to destroy many tissues of its host. Surface membrane interactions between *Entamoeba histolytica* and its host are of critical importance and an understanding of the biochemical basis for the parasite adherence and cytolytic activities are crucial for vaccine development (Radvin 1989 *J Infect Dis 4*: 1185-1207). Plasma membrane of cells contains enzymes whose active sites face the external medium rather than cytoplasm. The activities of these enzymes, referred as ecto-enzymes, can be measured using intact cells and can be modulated by divalent cations such as Mg^{2+} and Ca^{2+}. We have recently characterized an ecto-ATPase activity in another protozoan of genus *Leishmania* (Meyer-Fernandes et al. 1997 *Arch Biochem Biophys 341*: 40-46). Ecto-ATPases, Ecto-ATP-diphosphohydrolases or E-type ATPases are glycoproteins that hydrolyze extracellular nucleotide tri- and/or diphosphates. Here we shown that live *Entamoeba histolytica* is able to hydrolyze extracellular ATP. Cellular viability was assessed, before and after incubations, by eosin method. We observed that live cells of *Entamoeba histolytica* present a low level of ATP hydrolysis in the absence of divalent metal (65 nmoles Pi x h^{-1} x 10^{-5} cells). However in the presence of 5 mM $MgCl_2$ the ecto-ATPase activity was 280 nmoles Pi x h^{-1} x 10^{-5} cells. The Ecto-ATPase described here is also stimulated by $MnCl_2$ and $CaCl_2$ but not by $SrCl_2$, $ZnCl_2$ or $FeCl_2$. The activities were linear with the time until at least one hour and with the cell density. The Mg-independent ATPase activity did not change in the pH range from 6.4 to 8.4. However the Mg-dependent ATPase activity did increase with the increase in the pH. To confirm that the observed ATP hydrolysis was not promoted by neither phosphatase nor 5'nucleotidase activities, a few inhibitors for these enzymes were tested. Vanadate, levamisole, molybdate and NaF did not change the ATPase activities.

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BC-120
GENETIC POLYMORPHISM IN A CLONAL POPULATION OF *ENTAMOEBA HISTOLYTICA* ASSOCIATED WITH BACTERIA

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Phenotypic and genotypic variations have been documented in *Entamoeba histolytica* (strains and clones) but the mechanisms concerning these variations are still not understood. Non-reciprocal recombination has been propose as one of the molecular events that produces genotypic variability in this parasite. In this work we obtained a clone of *E. histolytica* from the axenic strain MAV-CINVESTAV, and trophozoites from this clone (clone MAVIII) were cultured in axenic conditions (MAVIIIax) or co-cultured with *Clostridium symbiosum* (MAVIIImx) or with *Pseudomonas aeruginosa* plus *Streptococcus faecalis* (MAVIIIpx). Clone A of *E. histolytica* (strain HM1:IMSS) was used as a control in all experiments.The genetic relationship between clones MAVIII and A was demonstrated by the gene signature of a 482 bp fragment from de M17 surface antigen gene using the Low-stringency Single Specific Primer PCR (LSSP-PCR) technique. Patterns obtained from LSSP-PCR were similar in all MAVIII cultures with only small differences in the intensity of some bands. Interestingly, although they shared some bands with clone A, their patterns differed. We detected several high molecular weight bands in clones MAVIII which were not present in clone A and low molecular weight bands in clone A that were not detectable in the clones MAVIII. The genotypic variability of clones MAVIII was also shown by the Random Amplified Polymorphic DNA (RAPD-PCR) technique using total DNA from the all cultures. We used six randomly selected primers, and although we could find shared bands among DNA from trophozoites of MAVIII cultures and clone A, all patterns differed among them. Our results indicate the occurrence of a high DNA poymorphism in *E. histolytica* strains, and also in a clone cultured under different conditions. Differences among the MAVIII cultures confirmed the plasticity of the genome of such parasite and its genotypic variability. The DNA polymorphism may be due to mutations, recombination, or amplification of certain DNA regions, that could be induced by the presence of the bacteria. Although we have evidences for DNA amplification in *E. histolytica*, the relationship between the polymorphism found in MAVIII clones and gene amplification remains to be investigated.

BC-121
INFLUENCE OF BACTERIA IN THE VIRULENCE OF A CLONAL POPULATION OF *ENTAMOEBA HISTOLYTICA*

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Entamoeba histolytica, the parasite responsible of human amoebiasis shows variability in various attributes such as virulence. The mechanisms that generate this variation are still poorly understood. In this work we present results concerning virulence analysis of clone MAVIII trophozoites obtained from axenic (MAVIIIax), monoxenic (MAVIIImx) and polyxenic (MAVIIIpx) cultures. The original polyxenic strain MAV-CINVESTAV was isolated from an asymptomatic carrier, and later axenized. We obtained the clone MAVIIIax by three subsequent cloning steps of the original axenic strain MAV-CINVESTAV. The clone MAVIIImx was originated by cultivation of the clone MAVIIIax for more than two years together with penicillin-inactivated *Clostridium symbiosum* in stationary phase. Subsequently, to obtain the clone MAVIIIpx MAVIIImx trophozoites were cultured in Pavlova's modified medium, supplemented with stationary cultures of two bacteria isolated from the original intestinal flora of the polyxenic strain MAV-CINVESTAV: *Pseudomonas aeruginosa* and *Streptococcus faecalis*. The axenic and monoxenic clones used in this work were cultured in TYI-S-33 medium. The first change observed in the trophozoites influenced by the presence of bacteria in the culture was a decrease in their cellular volume. The MAVIIImx trophozoites presented 35% while MAVIIIpx trophozoites presented 30% of the total cellular volume of the MAVIIIax trophozoites. To investigate whether the MAVIIImx and MAVIIIpx trophozoites had dependence on the bacteria present in their medium, we eliminated the bacteria by supplementing all MAVIII cultures with cefotaxime. The clone MAVIIIax, used as a control, and the clone MAVIIImx grew almost equally well in the presence and in the absence of the antibiotic, being that cefotaxime had a dramatic effect on growth of MAVIIIpx trophozoites, since they died after 24 h treatment. The virulence of the trophozoites was evaluated *in vitro* (erythrophagocytosis, cytopathic, and cytotoxic effect on MDCK cell monolayers) and *in vivo* (intraperitoneal inoculation in hamsters). MAVIIImx trophozoites presented virulence *in vitro* similar to one showed by the clone MAVIIIax, but MAVIIImx trophozoites did not produce hepatic abscesses in hamsters. By contrast, MAVIIIpx trophozoites showed a dramatic decrease in their virulence "in vitro" as "in vivo". These results showed that MAVIIImx trophozoites had little dependence on bacteria to grow, in contrast to MAVIIIpx trophozoites that showed a strong dependence on bacteria to survive. The here reported results seems to point out that bacteria are differentially associated with the trophozoites of a particular culture, selecting trophozoites with different phenotypes. Further, selection may occur also in the host, where the different intestinal flora could influence the degree of virulence of *E. histolytica*.

BC-122
IN VIVO RELEVANCE OF THE CYSTEINE PROTEINASE CP65 OF *TRICHOMONAS VAGINALIS* INVOLVED IN CYTOTOXICITY

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Trichomonas vaginalis a flagellated protozoan parasite is responsible for trichomonosis. The mechanism of trichomonal cytopathogenicity is a multifactorial process; but, the molecules involved in cellular damage remain poorly defined. Lately, parasite proteinases have received attention for their possible role in virulence properties i.e., cytoadherence, cytotoxicity, haemolysis, nutrient acquisition, immune evasion, etc. Recently, we have identified and characterized a 65 kDa cysteine proteinase (CP65) localized on the surface of *T. vaginalis* and with affinity to the host cell surfaces, which has been involved in trichomonal cytotoxicity. To asses the in vivo relevance of the CP65 proteinase we determined pH and temperature range of proteinase activity, immunogenicity and secretion into the vaginal environment. For that purpose, we analyzed twenty-eight human sera and forty-three vaginal washes by immunoprecipitation assays and substrate gel electrophoresis. The proteinase activity was observed at pH range from 4.5 to 7.0 at 37ºC temperature, similar to those observed in patients with trichomonosis. However, its proteinase activity was diminished or absent at temperatures below (4º to 35ºC) or above body temperature with a thermostability up to 60ºC. On the other hand, from the twenty-eight human sera studied, twenty-one (21/28) had diagnosis of trichomonosis, but only seven of them (7/21) were confirmed by *in vitro* culture. The other seven sera (7/28), four (4/7) were from patients with other STDs and three (3/7) from normal people. All patient sera with trichomonosis (culture-positive, 7/7) and only three (3/14) with clinical diagnosis showed anti-CP65 antibodies. Sera from normal people or with other STDS did not immunoprecipitate this proteinase. Twenty VWs from patients with diagnosis of trichomonosis were studied, only seven of them (7/20) were culture-positive and only these VWs (7/7) had the CP65 proteinase. As negative control, we used twenty-three VWs, fourteen (14/23) with other STDs and nine (9/23) from healthy people, neither one of them had the CP65 proteinase. The CP65 proteinase involved in trichomonal cytotoxicity has a proteinase activity at a pH range found in patients, is immunogenic, and is secreted into the vaginal environment indicating its *in vivo* relevance.

BC-123
HUMAN PATHOGENIC MICROSPORA: CHITINOLYTIC ENZYME ACTIVITY IN THE SPORES AND THEIR INHIBITION BY CHITIN HYDROLYSATE

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The world-wide distributed phylum *Microspora Sprague*, 1977 (Protozoa) contains more than 140 genera and more than 1000 species. These Protozoa, distributed by spores with a chitinous spore wall are obligate intracellular parasites in man and animals. The genera *Nosema, Vittaformis, Pleistophora, Trachipleistophora, Encephalitozoon, Enterocytozoon* and the collective group *Microsporidium* include human pathogenic species which are able to induce in man a lot of different clinical manifestations. For the infection of host cells, the coiled polar tubules has to pass through the chitinous spore wall, but nothing is known about this mechanism. Therefore we have looked for the presence of a chitinolytic enzyme activity in the spores of human pathogenic species.*Encephalitozoon cuniculi* and *E.intestinalis* were kept in tissue culture (E6 green monkey kidney cells) at 37ºC. From the culture supernatant the spores were harvested (l5OOxg/2O min.) and thrice washed in PBS pH 7.2. The substrate for the detection of the chitinolytic enzyme activity was 4-methylumbelliferyl-b-D-N,N',N", triacetylchitotriose (4-MU$(GlcNAc)_3$) (Sigma, Gernany) in a final conc. of 7.5/ug/ml DMSO/PBS= (sol.3). To take the fluorescence, the assays were carried out in Quartz 1/2 micro cuvettes and the fluorescence was performed in a SFM 25 Kontron Spectralfluorimeter (280 nm - 800 nm)(Kontron, Switzerland; excitation 355 nm, emission 460 nm; high voltage 340).Two test groups (A,B) were carried out: Test A (I,II,III) has a constant spore concentration of $7xl0^7$ spores and a variable sol.3 volume (1.8ml; 0.9ml, 0.45ml) and Test B (I,II,III) has a constant sol. 3 volume (1.8ml) and a variable conc- of spores ($1.1Ixl0^8$, $5.5x10^7$; $2.7xl0^7$). To get the null value the Eppendorf tubes were put on ice. After printing the fluorescence units (FU), the test tubes were incubated at 37ºC water bath and in 15 min intervals the FUs were taken up to 120 min. The test AI was also carried out at 4º C and with spores incubated for 15 min at 55ºC. Furthermore it was tested whether chitin hydrolysate (Dunn, Germany) can be used as inhibitor for the chitinolytic enzyme activity of the spores. $1x10^7$ spores were incubated in 250ml buffer plus 250ml. chitin hydrolysate plus 1.8 ml sol-3. The run of the graphs (curvilinear) of the tests A(I,II,III) and B(I,II,III) demonstrates the presence of a chitinolytic enzyme activity in the spores of Microspora; this enzyme activity can be inhibited by chitin hydrolysate; the chitinilytic enzyme activity is blocked at 4º C and inactivated by incubation of the spores at 55ºC. We think that this chitinolytic enzyme activity plays a part in the extrusion mechanism of the polar tubulus.

BC-124
GLYICOLIPID IN NONPATHOGENIC TRYPANOSOMATID

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The genus *Crithidia*, a human nonpathogenic flagellate, is a representative of the Tripanosomatidae family. These organisms present a highly glycosilated membrane containing glycolipids and glycoproteins. That are believed to be involved in several membrane-mediated phenomena. In *Tripanosoma cruzi* Y strain the major glycosphingolipid fractions comprises only mono- and dihexosides (Barreto- Bergter et al. 1992 *Mol Biochem Parasitol 51*: 263-270). In this paper, we described the isolation and identification of neutral glycosphingolipids from *Crithidia* species. Glycosphingolipids were extracted from cells and were purified by silica gel and Iatrobead column chromatography. These structures were determined by high performance thin layer chromatography (HPTLC), gas chromatography/mass spectrometry (GC-MS) and fast atom bombardment mass spectrometry (FAB-MS).

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BC-125
GIANT PROTEINS ASSOCIATED WITH THE CYTOSKELETON OF TRYPANOSOMATIDS PROMASTIGOTE FORMS: DIFFERENCES IN BIOCHEMICAL AND IMMUNOCYTOCHEMICAL PROPERTIES

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The cytoskeleton of eukaryotic cells has been shown to operate in important functions such as cell movement, intracellular transport, division and the control of cell shape. The trypanosomatids have a very precisely ordered microtubule cytoskeleton and represent one the most interesting biological model for the study of the biochemistry, organisation and function of proteins. In all genera of the trypanosomatids (*Phytomonas, Leptomonas, Trypanosoma, Crithidia, Leishmania, Herpetomonas* and *Blastocrithidia*) we have identified a novel class of megadalton proteins associated with their cytoskeleton. These proteins present molecular masses ranging from 1000 to 3500 kDa, and different immunochemical properties. Herein, we shall discuss the differences in the biochemical and immunochemical properties of these proteins as well as the subcellular localization in promastigote forms of *Leishmania tarentolae, Leptomonas samueli* and *Phytomonas serpens.*

Both Indirect Immunofluorescence and immunogold electron microscopes analysis revealed that in Leishmania, the giant protein is distributed along the flagella, whereas in *Phytomonas*, it is essentially located in the FAZ (flagellar attachment zone). However, in *Leptomonas*, both the FAZ and the whole cell body skeleton are labeled. Confocal images and biochemical analysis of *L. samueli* and of *P. serpens* demonstrated that their giant protein appear to interact with the cell body microtubules. These results show, for the first time, the existence of megadalton proteins presenting properties similar to MAPs (microtubules associated proteins). Moreover, for each genera of promastigote forms of trypanosomatids, these proteins appear to play a specific structural function in the cytoskeleton.

BC-126
CHARACTERIZATION OF A CA^{2+} TRANSPORT IN MEMBRANE VESICLE PREPARATIONS FROM *PHYTOMONAS SERPENS*

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There is compelling evidence for the role of Ca^{2+} ions as intracellular messengers in a number of different cellular functions. Evidence is accumulating in support of an important role for Ca^{2+} in trypanosomatids. Thus, microtubule assembly in *Trypanosoma brucei*, flagellar movement in *Crithidia oncopelti*, and cellular differentiation in *Leishmania*, appear to be regulated by Ca^{2+}. In most eukaryotic cells, Ca^{2+} homeostasis is achieved by the concerted operation of several Ca^{2+}-transporting systems located in the plasma membrane, endoplasmic reticulum and mitochondria. In this work, we investigated the presence of a Ca^{2+}-ATPase in membrane fractions of *Phytomonas serpens* promastigotes, a parasite which belongs to the Trypanosomatidae family, that is commonly isolated from plants and phytophagous insects. The membrane vesicles were prepared essentially as reported by Benaim et al. (1991 *Biochem J 280*: 715-720). The vesicles showed Ca^{2+}-ATPase activity (0.30 mmol $Pi.mg^{-1}.45min^{-1}$) at room temperature (25-30°C) in the presence of 1 mM $MgCl_2$ and 1 mM ATP. Optimal activity was observed at pH 7.0 . It was measured in the presence of azide, ouabain and KNO_3, to avoid the action of other ATPases. The ATPase stimulated by Ca^{2+} was inhibited by 1mM vanadate. Ca^{2+} uptake observed in the presence and absence of potassium oxalate was 20 nmol$Ca^{2+}.mg^{-1}.h^{-1}$ and 3.5 nmol $Ca^{2+}.mg^{-1}.h^{-1}$, respectively. Other nucleotides like GTP, UTP and ITP could replace ATP in promoting Ca^{2+} uptake, but with much less efficiency. Thapsigargin (1mM), cyclopiazonic acid (2mM), bafilomycin A_1 (50 nM) and myo-inositol 1,4,5-trisphosphate (4 mM) did not inhibit the Ca^{2+} uptake. Inhibition by vanadate showed two populations of enzymes, one more sensitive (Ki < 25 mM) and another one less sensitive (Ki @ 1 mM). These results suggest that more than one active transport of Ca^{2+} may be involved in the regulation of the cytoplasmic Ca^{2+} levels. This microsome preparation we characterized could be a good model for the study of this regulation.

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BC-127
FUNCTIONAL STUDIES OF SEQUENCES WITH HIGH PROPENSITY TO FORM G-QUARTET STRUCTURES FROM MINICIRCLES OF *PHYTOMONAS SERPENS*

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We have previously sequenced minicircles from *Phytomonas serpens* and found telomere-like sequences rich in G and T. These were shown to be capable of forming G-quartet structures *in vitro*, in the absence of proteins. Many theories have been proposed for a biological role for G-quartet structures. They might have a role in chromosome pairing and in HIV they might be involved in the association of the two homologous RNA strands. Also, the biological relevance of these structures has been stressed by recent isolation of protein activities clearly related to G-quartets. We are presently investigating a putative biological role for these sequences in trypanosomatid kinetoplast. In order to verify a possible interaction of these GT-rich sequences with proteins, we are carrying on mobility shift studies. We have isolated a kDNA-protein enriched fraction in discontinuous gradients. When the proteins were examined by PAGE, a prominent band was visible at approximately 50kDa. This fraction is being incubated with synthetic oligonucleotides containing either the GT-rich sequence or the control complementary strand, preincubated in conditions of G-quartet formation, and examined in non-denaturing gels.

We are also investigating a possible specific location of these sequences in the kinetoplast, that might indicate a structural function. Biotinylated GT-oligonucleotides were hybridized *in situ* to *Phytomonas serpens* and examined by electron microscopy. Preliminary results indicates a location in the borders of the kinetoplast.

BC-128
EXTRACELLULAR PROTEOLYTIC ACTIVITIES IN THREE *PHYTOMONAS* SPECIES DIFFERING IN HOST SPECIFICITY

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Flagellate trypanosomatids of the genus *Phytomonas* are etiological agents of diseases affecting fruits and plants of great economical importance, although they also parasitize many plants without apparent pathogenicity (Dollet 1984 *Annu Rev Phytopathol 22*: 115-132). In this study, extracellular proteinases of three phytoflagellates, *Phytomonas françai*, *Phytomonas serpens* and *Phytomonas* sp. from different hosts including cassava, tomato and milkweed plant *Euphorbia hyssopifolia*, respectively, were analysed. Cells were cultivated in BHI at 28º C. Cell-free supernatants were obtained after centrifugation of the cultures at the end of log phase. Proteolytic activity was determined by SDS-PAGE containing co-polimeryzed gelatin as substrate (Heussen & Dowdle 1980 *Anal Biochem 102*: 196-202). The gels were incubated at 37º C at pH 5,5 and at pH 10. Additionally, a quantitative analysis was assayed using azocaseine as substrate (Guinter 1979 *Antimicrob Agents Chem 15*: 522-526) for proteolytic activity and BSA for determination of protein content (Lowry et al. 1951 *J Biol Chem 193*: 265-275). SDS-PAGE analysis revealed the presence of at least four bands at 94 kDa, 75 kDa, 67 kDa and 40 kDa, in the culture supernatant of *P. serpens* and *Phytomonas* sp. in both pH 5,5 and pH 10, and one band at 30 kDa in *P. françai*. These results are correlated with the azocaseinase assay in which *Phytomonas* sp and *P. serpens* extracellular proteinases displayed more activity than *P. françai*. Studies with proteolytic inhibitors are in progress in order to determine the enzymatic class of these enzymes.

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BC-129
DISTINCT PROTEINASE PROFILES DISPLAYED BY TRYPANOSOMATIDS HARBORING OR NOT AN ENDOSYMBIONT

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In the trypanosomatids, proteinases have been implicated in host-parasite interactions and in the processing of proteins for nutrition purposes (McKerrow et al. 1993 *Annu Rev Microbiol 47*: 821-853). Some trypanosomatid species have an endosymbiont in their cytoplasm that probably provides essential nutrients to and induces morphological alterations in the protozoa (Motta et al. 1997 *Eur J Cell Biol 72*: 370-377). Here, we presented a comparison between cellular and extracellular proteolytic activities in trypanosomatids with endosymbiont or not. We surveyed the species *Crithidia deanei*, *Crithidia desouzai* and *Herpetomonas roitmani*, endosymbiont-bearing trypanosomatids,

and *Crithidia fasciculata* and *Herpetomonas megaseliae* by the rapid detection method of SDS-PAGE gels containing co-polymerized gelatin as substrate (Heussen & Dowdle 1980 *Anal Biochem 102*:196-202). Cells were grown during two days at 28ºC. After centrifugation, 10^8 cells were lysed by the addition of SDS-PAGE sample buffer (Laemmli 1970 *Nature 227*:680-685), and the culture supernatant equivalent to 10^9 cells was concentrated against polyethyleneglycol before the addition of sample buffer. A similar profile was detected in endosymbiont-bearing species: in the culture supernatant, two proteolytic enzymes were observed migrating at 85kDa and 60kDa, while cell-associated proteinases migrated in the 45 kDa-70kDa range. The comparison to species of the same genus without endosymbiont revealed qualitative and quantitative differences. Besides the proteinases described above, a 110kDa band was detected in *C. fasciculata* culture supernatant. A related profile was already described for *C. guilhermei* (Melo et al. 1998 XXVII SBBq Meeting, p. 104). In *H. megaseliae*, lower molecular mass proteinases were detected at 35kDa and 40kDa. Secretion of proteolytic enzymes is apparently diminished in endosymbiont-bearing species. In all samples, the enzymes appear to be metalloproteinases since 1,10-phenanthroline inhibited them. Some differences could also be observed when cell-associated proteinases were analyzed. *C. fasciculata* and *C. guilhermei* proteinase profile comprises at least three bands in the 55-105kDa range (Branquinha et al. 1996 *J Euk Microbiol 43*:131-135). In *H. megaseliae* cells, the four major proteolytic activities were detected between 35kDa and 85kDa. Taken together, these observations suggest that alterations in the proteolytic profile could be associated to the presence of an endosymbiont in some trypanosomatid species.

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BC-130
CHROMATIN AND HISTONES IN *GIARDIA LAMBLIA*

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The human enteric protozoan, *Giardia lamblia*, the etiologic agent of giardiasis, has generated much interest because it constitutes the earliest branching lineage among eukaryotes, and may represent a missing link in the transition from prokaryote to eukaryote. Prokaryotes do not have histone proteins. For that reason the knoledwge of the histone proteins and the structure of chromatin in Giardia, are interesting because they may represent preliminary stages in the formation of modern nucleosome and histones. As a contribution to the understanding of *G. lamblia* chromatin organization, in this work we characterize the histone proteins of the parasite using two electrophoretic systems: SDS-PAGE and TAU-PAGE geles. Eight proteins displaying similar characteristic to histones have been observed. Two proteins, named as G1 and G2, migrate slower than histone H2A of the controls: *T. cruzi* and calf thymus. Proteins G5, G6, G7 and G8, migrate in the region of standar nucleosomal core histones, but were distinguishable. This pattern indicate that the histones of *G. lamblia* are divergent in relation with the histone of Trypanosomatids and higher eukaryotes. On the other side, electron microscope studies of *G. lamblia* chromatin, indicate that its DNA is organizated in nucleosomes. Sequence analysis of *G. lamblia* histones, which were purified by HPLC, will permit quantificate the histone divergence of this parasite and postulate an evolutive history of the histone and nucleosome.

BC-131
BIOCHEMICAL CHARACTERIZATION AND CYTOCHEMICAL LOCALIZATION OF AN ECTO-ATPASE IN *CRITHIDIA DEANEI*

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Crithidia deanei is a trypanosomatid which harbours an endosymbiotic bacteria in the cytoplasm. A true symbiont relationship is maintained since only 1 to 2 microorganisms are found per host cell. Symbionts furnishe aminoacids, vitamins and purins to the protozoa and are also capable of inducing morphological and physicochemical chances in the host. Here, we report on the presence of an ATPase activity stimulated by $MnCl_2$ ($S_{0.5}$ = 0,25 mM) or $MgCl_2$ ($S_{0.5}$ = 0,56 mM), but not by $SrCl_2$ ou $CaCl_2$. This ATPase activity is located on the external surface of *C. deanei*, which was supported by its sensivity to impermeant reagents, such as DIDS (K_i » 70 mM) and the observation that inhibitors of other ATPases, with internal ATP binding sites, did not change the activity of this enzyme. We measured nucleotidase and phosphatase activities using 5'-AMP, 5'-ADP or pNPP as substrates. These activities were not stimulated by $MgCl_2$, suggesting that the ATP hydrolysis was due to ecto-ATPase activity. We observed that increasing concentrations of suramin, a potent non-selective P_2 antagonist, inhibits progressively the ecto-ATPase activity of *C. deanei*. We also noticed that the cured strain (antibiotic-treated) showed a lower Mg^{2+}-dependent ATPase activity when compared with the normal strain, suggesting that the endosymbiont may induces

the expression of this enzyme. Ultrastructural cytochemistry was employed to localize the Mn^{2+}-ATPase activity. Labeling was detected at the protozoa plasma membrane in a heterogenous way, since reaction product was seen only in some points of the cell surface and never at the flagellar pocket membrane. The physiological role of this enzyme is still unknown, however several hypotheses have been suggested as protection from cytolytic effects of extracellular ATP and involvement in signal transduction.

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BC-132
A STUDY OF EXTRACELLULAR PROTEINASES FROM *CRITHIDIA GUILHERMEI*

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Crithidia spp. are monogenetic trypanosomatids that display a characteristic choanomastigote form and are non-pathogenic to humans. These trypanosomatids secrete proteinases, that are enzymes implicated in a broad spectrum of important biological reactions such as protein metabolism, immune reactions, intracellular survival and escape (McKerrow et al. 1993 *Annu Rev Microbiol 47*: 821-853). In this work, a partial purification of extracellular proteinases from *C. guilhermei* and the proteinase production in different media were reported. The microorganism was grown in yeast extract-peptone-sucrose medium during three days at 28°C. The culture supernatant was harvested by centrifugation at 3000 rpm during 15 min, and it was precipitated using ammonium sulphate at 40% saturation. The precipitated proteins were resuspended and dialysed against PBS. The proteolytic activity from each fraction was assayed with azocasein as substrate (Ginther 1979 *Antimicrobiol Agents Chem 15*: 522-526) and the total protein concentration was determined by the method of Lowry et al. (1951 *J Biol Chem 193*: 265-275). In order to verify the proteinase production on different media, the trypanosomatid was also grown in two different media besides yeast extract-peptone-sucrose: yeast extract-peptone-glucose and yeast extract-peptone-glycerol. The proteolytic activities were detected on 10% SDS-PAGE containing gelatin as substrate (Heussen & Dowdle 1980 *Anal Biochem 102*:196-202). In this step in the purification procedure, the enzyme was purified 14-fold. In the two latter media tested, the proteinase production was the same with three bands being detected migrating at 67-43 kDa range; only in yeast extract-peptone-sucrose four bands were observed at 94-43 kDa range. Other purification procedures and characterization of these enzymes are in progress.

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BC-133
A FURTHER CHARACTERIZATION OF NOVEL ECTO-ATPASE ACTIVITIES PRESENT ON THE SURFACE OF *TRITRICHOMONAS FOETUS*

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Tritrichomonas foetus is a parasite of the bovine urogenital tract which shows great veterinary and economical importance, as this flagelate is the etiologic agent of bovine sexually transmitted trichomoniasis. Ecto-ATPases are glycoproteins that hydrolize extra nuclear nucleotides tri and/or diphosphates. We have recently characterized an ecto ATPase activity in *Leishmania* (Meyer-Fernandes et al. 1997 *Arch Biochem Biophys 341*: 40-46,), which share some common features with those here described. In this work we identified two ecto-ATPase activities in alive *T. foetus*, one is Mg^{2+} dependent and another one which is independent on Mg^{2+}. The cell viability was inferred by motility and Trypan blue dye exclusion and the localization on the exterior side of the membrane by several tests including sensitivity to the impermeant agent DIDS. Both enzymes presented broad specificity to triphosphate nucleosides (ITP, CTP, UTP and GTP), but preferentially hydrolized ATP. The Mg^{2+}-dependent ecto-ATPase was more active at pH 8.0, while the best activity for the Mg^{2+}-independent enzyme was at pH 6.4. Concanavalin A, *p*-NPP, Pi, 5'-AMP, adenosine, levamizole (alcaline phosphatase inhibitor) and NaF (phosphatase inhibitor) had little or no effect on those enzyme activities. The Mg^{2+}-dependent ATPase presented two different K_m values for the substrate ATP: K_m 1 = 0.01 mM and V_{max} 1 = 0.16 nmol Pi / min / 10^7 cells; K_m 2 = 7.27 mM and V_{max} 2 = 1.79 nmol Pi / min / 10^7 cells (n2 = 1.99 and $K_{0.5}$ 2 = 2.71). In order to test the possibility that *T. foetus* was capable of secreting soluble enzymes to the external medium, the culture medium was tested for ATPase activity, which was found to be 28.2 nmol Pi/h, on the exponential phase of growth (24 hr). This suppernatant showed hydrolitic activitiy for the substrates ATP, ADP, *p*-NPP and 5'-AMP. Nevertheless none of these activities were stimulated by Mg^{2+}.

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BC-134
USE OF PROBES (SL201, SL3', SSU3) AND SPECIFIC PRIMER (PCR)IN THE CHARACTERIZATION OF TRYPANOSOMATIDS ISOLATED FROM PHYTOPHAGOUS HEMIPTERA

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The Trypanosomatidae family are largely distributed in the nature and can be found in vertebrate, invertebrate and plants. The taxonomy of trypanosomatids, specially the *Phytomonas* genus is complex and constitutes a problem in the study of these parasites. The systematic determines that all trypanosomatids isolated from plants are considered *Phytomonas*, however this criterion is not sufficient because the genera *Crithidia, Leptomonas* and *Herpetomonas* also were detected in fruits and grains. Some genera can be determinate by the presence or absence of arginase enzyme in urea cycle. This enzyme can be found in the genera *Crithidia* and *Leptomonas* whereas in the genera *Phytomonas* and *Herpetomonas,* the arginase is not present. This criterion in association with other tests involving physiologic, immunologic, genetic and morphologic aspects can be appropriate for the characterization and identification of trypanosomatids. Teixeira et al. used the miniexon genes of characteristic organization of RNA spliced leader with success to characterize some trypanosomatids isolated from plants and insects. In this study, we used the probes SL201 (recognizes Trypanosomatidae), SL3 (recognizes *Phytomonas*), PCR (to confirm *Phytomonas* genus) and SSU3 (indicated for identification of some species of *Herpetomonas* and *Leptomonas* – absence of arginase activity) to characterize 47 strains of trypanosomatids isolated from phytophagous hemiptera, previously characterized by the enzymatic profile of urea cycle. The results showed 100% (47) of hybridization with SL201 probe indicating that all strains are Trypanosomatidae; 14.9% (7) were positive to SL3 probe and these same strains (7) were confirmed with PCR as being *Phytomonas* genus, and 87.7% (40) of the strains that presented enzymatic profiles of urea cycle similar to those of *Herpetomonas*, *Leptomonas* or *Crithidia* genera were confirmed not belonging to *Phytomonas* genus. Only one strain (710TD) presented hybridization with all probes suggesting the necessity of a new clonage to obtain a uniform populations. Our results demonstrated that SL201 and SL3'or specific primer(PCR) to *Phytomonas* genus were useful to characterize trypanosomatids with promastigote forms of the genus *Phytomonas*.

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BC-135
POLYMORPHISM IN THE SECOND INTERNAL TRANSCRIBED SPACER (ITS2) OF RIBOSOMAL DNA AMONG POPULATIONS OF *ANOPHELES OSWALDOI*

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Recente data have appointed *Anopheles oswaldoi* as an important malaria vector in localities os Peru and Acre (Brazil). However, in Rondônia, only a very low number of *An. Oswaldoi* fed on malaria patients developed salivary gland infections. These data, together with the difficulties found to identify anophelines of the Oswaldoi Group based on morphologic criteria suggest that specimens os *An. Oswaldoi* are member of a complex e closely related species. The distincton of sibling species of insects is of criticalimportance, since different member in a Complex could exhibit differences in ecology , vectorial capacity and response to control measures. DNA sequence analysis and particularly that of the ITS2 region rDNA cistron has provided diagnostic characters in some group of sibling species becoming a general tool for taxonomic and phylogenetic studies. The present study was undertaken to determine the extent of differences over the ITS2 sequence of specimens of *An.oswaldoi* captured in several localities of South América. The ITS2 of these anophelines were amplified using conserved primers of the 5.8S and 28S regions, cloned and sequenced. The lengths of ITS2of all mosquitoes captures were about 350 nucleotides , with about 53% GC content. Analysis of the alignment of the sequences, wich showed that the similarity varied between 87% and 100% and analysis of a neighbor-joining tree produced with p-distance using ITS2 sequences, separated these specimens in four groups. One of them is probably related to *An.oswaldoi* sensu stricto, and another one can possibly be related to *An.Konderi*, since only morphological features in the male genitalia can distinguish these taxa. The other two groups may correspond to species wich morphologycal identification remanin to be clarified in the *An.oswaldoi* complex. These data are evidences that specimens of *An.oswaldoi* are included in a complex of cryptic species and that the DNA identification could solve some taxonomic questions.

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BC-136
COMPARATIVE STUDY OF THE DIAGNOSIS OF AMERICAN CUTANEOUS LEISHMANIASIS BY PCR USING TISSUE BIOPSIES AND IMPRINT OF THE BIOPSIES ON FILTER PAPER, AS PARASITE DNA SOURCE

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Thirty-five human patients with dermal lesions compatible with American Cutaneous Leishmaniasis, were attended at the Leishmaniasis Ambulatory from Caratinga, Rio Doce Valley, Minas Gerais State, Brazil. Skin biopsies of approximately 5 mm diameter were taken under sterile conditions from the border of the ulcers in each patient. The parasitological diagnosis was made on Giemsa-stained smears of the patient's biopsies. Amastigotes (AMA) were quantified in 100-250 mononuclear cells (MC)/slide. The parasitism was considered low and high when the percentage of AMA/MC was lower or higher than 5% respectively. Twenty two out of 35 (62.9%) patients were parasitologically confirmed. From those, 11 presented low parasitism and 11 high parasitism. For Polymerase Chain Reaction (PCR) primers designed to amplify the conserved region of the minicircle from the *Leishmania* kDNA were used (Passos et al.,1996). The DNA samples were extracted from biopsies conserved on 70% ethanol and from biopsies imprinted on filter paper. The reason for using filter paper imprint is its suitability for field work. The PCR results obtained with the DNA extracted from filter paper showed that 19 were positive out of 22 patients parasitologically confirmed (86.4% sensibility) and 3 out of 13 parasitologically negative patients gave PCR positive (76.9% specificity). The PCR results with DNA extracted directly from biopsies showed that 21 were positive out of 22 patients parasitologically confirmed (95.5% sensibility), and 2 out of 13 parasitologically negative patients gave PCR positive (84.6% specificity). We think that the two patients that gave PCR positive using DNA from biopsies and negative using DNA from filter paper, were due to excess of blood on the imprints. In conclusion, the PCR using tissue biopsies as *Leishmania* DNA source presented a better performance than that using filter paper. Further improvements should be made on DNA extraction from filter paper in order to reach a better PCR performance.

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BC-137
PANCREATITIS INDUCED BY SODIUM STIBOGLUCONATE (SB^V) IN BALB/C TREATED MICE AFTER INFECTION WITH *LEISHMANIA (LEISHMANIA) AMAZONENSIS.* COMPARISON WITH MEGLUMINE ANTIMONATE THERAPY

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Sodium Stibogluconate (Sb^V) and Meglumine Antimonate (Glucantime®) are the first choice drugs against all forms of Leishmaniasis. In Brazil, a Chinese formulation of Sb^V (BP88®) was imported for the therapy of *Leishmania* infections in endemic areas without previous studies. The aim of the present work was to study the efficacy and toxicity of the Chinese Sb^V as compared to Glucantime®, in the treatment of experimental infections of Balb/c mice with *L. amazonensis*. Thirty seven female Balb/c mice were infected in the left hind footpad with 5×10^6 promastigotes of the MHOM/BR88/BA/125 strain and divided in 3 groups: G1: 18 mice treated with 100ml (10mg) Sb^V /day; G2: 9 mice treated with 100ml (~10mg) Glucantime®/day; G3: 10 mice treated with 100 ml saline/day. Therapy was initiated 2 weeks after infection and injections were made by the intraperitoneal route for 21 days. Assessment of lesion development was made weekly by direct measurement of both hind footpads with a dial gage caliper. Size of the lesions was determined by the delta (D) value between the normal right footpad and the inoculated left footpad. The M and SD values were calculated and plotted against time of observation. All mice developed lesions at the site of inoculation regardless of the treatment applied. Only during the therapy was regression of the lesions observed. Analysis of the rate of lesion development showed a decreased variability in the Sb^V treated group. Nevertheless, mice treated with Sb^V were visibly more debilitated and had an episode of diarrhea when a second course of treatment was tried. Histopathological examinations of sections of heart, lungs, liver, kidneys, pancreas, spleen, intestines, popliteal lymphnode and infected footpad were made by H x E staining. Pancreatitis was observed in 17 of the 18 mice treated with Sb^V. The inflammation had a focal aspect but in most cases was disseminated througout the organ. No pancreatitis was seen in mice of the Glucantime® treated and control groups.

BC-138
PHYSICAL MAPPING OF *LEISHMANIA MAJOR*: A TOOL AND SOME FINDINGS

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One of the major achievements of Genome Projects is the generation of tools for further studies to understand organization and dynamics of genomes, localizing not only genes but also structural sequences such as telomeres and centromeres. Physical mapping consists of ordering cloned genomic DNA in their original organization onto chromosomes. It can be pursued with different strategies and represents an important step in genome research. It is possible to generate a physical map using a chromosome specific or a random mapping strategy. The chromosome specific strategy has been carried out combining chromosome walking and non-random STS-content mapping procedures. The walking procedure is time consuming and inefficient to generate a map of the genome as whole, but originates fine maps and constitutes ready-to-use data for the study of many aspects of chromosome structure and genomic organization, presence and distribution of reiterate sequences. We have used ESTs (*e*xpressed *s*equence *t*ags) to map genes onto genomic clones as an approach to link physical and genetic mapping information and to generate a low resolution physical map randomly across the genome. These ESTs are used in hybridization experiments with the *L. major* - LV 39 (LV39, Rho/Su/59/P) arrayed genomic library. Besides identification of positive recombinant clones, each EST is hybridized to the chromosomes of *Leishmania* separated by pulsed field gel electrophoresis (PFGE). Expressed tags and recombinants are thus grouped according to chromosomal bands. A number of contigs have been produced for the LV 39 genome, the contigs and some analyses of the process will be discussed. The combination of both chromosome specific and global approaches allowed the confirmation and improvement of contigs generated for the mini-exon chromosome. The chromosome 2 map is complete and includes the telomeric end. This map contains a clone carrying only the miniexon array, which was transfected into an avirulent line of *L. major* to test wether the number of copies of the array would alter the pattern of virulence as suggested previously by Samaras and Spithill (1986 UCLA Symposia on *Mol Cell Biol 42*: 269-278). Only one transfectant (D2 clone, among four) showed significant increase in virulence *in vivo* when they were injected on susceptible animals (BALB/c). To test whether this effect was related to the miniexon extracopies, transfectants were cured. *In vivo* infection experiments have shown that the virulence observed in D2 clone is not related to the increased number of miniexon array. When D2 clone was kept under increasing drug concentration (40 and 80 :g/ml of hygromicin B) we observed a significant decrease in virulence.

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BC-139
ROLE OF PROTEASOME IN THE LIFE CYCLE OF *TRYPANOSOMA CRUZI*

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

BC-140
STANDARDIZATION OF CONDITIONS TO OBTAIN AMASTIGOTES FROM DIFFERENT *LEISHMANIA* SPECIES

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Leishmaniasis include a spectrum of diseases which results from states ranging from hipersentivity to anergy to parasite antigens. Although many factors influence the type of immune response to *Leishmania*, some species of the parasite such as *L. chagasi* and *L. amazonensis* are associated with anergic forms of disease, while *L. braziliensis* usually leads to delayed type hipersensitivity. The availability of pure amastigote preparations from different *Leishmania* species would facilitate studies aimed at identifying the *Leishmania* molecules involved in modulation of the infection. Preliminary data on the production and partial characterization of the antigens of amastigotes of *L. chagasi*, *L. braziliensis* and *L. amazonensis* are presented herein. Different conditions for mass production of amastigotes of *L. chagasi*, *L. amazonensis* and *L. braziliensis in vitro* are tested. Using the J774 macrophage cell line, 90 to 95% of

infection with the different species of *Leishmania* were obtained, with 5 to 25 amastigotes by macrophage. The viability of the parasites was however low specially after 48 hours of infection. The production of amastigotes from axenic cultures proved to be a better alternative. The different species of *Leishmania* required specific conditions of pH and temperature for amastigote transformation. The rate of amastigote transformation from promastigotes in axenic cultures were 91% for *L. chagasi*, 97% for *L. braziliensis* and 97% for *L. amazonensis*. The amastigotes produced in axenic cultures were well preserved at the ultrastructural level. They also maintained their ability to infect J774 cell line and revert to promastigotes under suitable conditions. The analysis of molecular profile through western-blot using a panel of polyclonal anti-*Leishmania* serum revealed a 44 kD band present only in amastigote, as compared to promastigote, lysates.

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BC-141
SIALYLATION PROTECTS *TRYPANOSOMA CRUZI* AGAISNT TRYPANOCIDAL ACTIVITY OF NITRIC OXIDE *IN VITRO*

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In the presence of sialic acid donors, *Trypanosoma cruzi* trypomastigotes acquire up to 10^7 sialic acid molecules per parasite. The sialic acid is incorporated in mucin-like glycoproteins that form a protective surface coat around the parasite surface. As Chagasic patients, and infected animals, present antibodies that inhibit trans-sialidase activity and prevent the sialylation of live parasites; these antibodies could contribute to elimination of blood trypomastigotes by immune system. Here we investigated whether sialylation could also protect trypomastigotes from killing agents found in the intracellular milieu. Typomastigotes were obtained from infected mammalian cells grown in the absence of sialic acid sources. The parasites were sialylated by addition of sialyllactose to compare the susceptibility to different killing agents. Resistance to acid pH and hydrogen peroxide was unaffected by sialylation. In contrast, sialylated parasites were more resistant to the killing mediated by S-nitroso-acetyl-penicillamine (SNAP) or sodium nitroprusside (SN), agents that generate NO *in vitro*. Non-sialylated trypomastigotes were killed at much lower NO donors concentrations when compared with sialylated parasites. This data suggests that in vivo inhibition of parasite sialylation by anti-trans-sialidase antibodies can contribute to the elimination of parasites, and that protection induced by immunization with trans-sialidase can also be mediated by conferring a higher susceptibility to NO inside macrophages.

BC-142
CLONING AND CHARACTERIZATION OF A *TRYPANOSOMA CRUZI* PROTEIN RICH IN GLUTAMIC ACID

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In the context of the *Trypanosoma cruzi* Genome Project of CYTED we sequenced an EST (expression site tag) of 718 bp. We screened a cosmid genomic library of the Genome Project using this marker as a probe. From 40,000 cosmids we isolated one, that was subcloned. Overlapping three subclones sequences allowed to identify the gene encoding this EST. This gene has an open reading frame for a glutamic and aspartic acid rich protein of 1,200 aa that we named *garp*. This protein doesn’t present homology with any other published in GenBanks. To find the trans-splicing site of this gene, we performed RT-PCR. Contrary to what we would predict, the 5’ splice acceptor site is located in the third AG after a pyrimidine rich sequence. Hybridization of Southern blots of digested genomic DNA suggested that there is only one copy of the gene per haploid genome. In accordance, the *garp* gene probe hybridized only with the XIX chromosomal band of *T. cruzi* CL-Brener. To complete the characterization of this protein, we tried to express it in bacteria. However, this was not possible due to its very long glutamic rich domain. Accordingly, we are producing anti-GARP polyclonal antibodies by immunization with a recombinant encompassing the N-terminal 300 residues of the GARP protein, fused to MBP (maltose binding protein). This construction excludes the negatively charged aminoacid domains. This and other GARP derived recombinant proteins will be expressed to study its antigenicity in Chagas disease.

BC-143
IN VITRO INHIBITORY ACTIVITY OF AJOENE ON ISOLATES OF *MICROSPORUM CANIS*

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Ajoene [(E,Z)-4,5,9-trithiadodeca-1,6,11-triene-9-0xide] is an organosulphur compound derivated from garlic (*Allium sativum*) with antiviral, antithrombotic, antiparasite and antifungal activities. We have determined the inhibitory activity of this compound on the *in vitro* growing of three isolates of *M. canis* coming from patients with dermatophytosis. Sabouraud medium was used either to adapt or to grow the isolates. The isolates were mantained on the exponential phase of growth at room temperature, with mechanical agitation (broth medium). A qualitative assay using agar plates was used to screen the sensitivity of the isolates to Ajoene and griseofulvine, ketoconazole and itraconazole, included as control drugs. We obtained the Minimal Inhibitory Concentration (MIC) and the Inhibitory Concentration 50% (IC50) using Optical Densities of the fungal homogenates grew in broth medium, in presence or not of Ajoene. A directly proportional increase of inhibition growth area related to Ajoene concentration was observed. When broth medium assay was used, a very strong dose-dependent effect was noted. The MIC values obtained were 10 µM for two of the isolates and 3 µM for the third one. On the other hand, the IC50 values were 1.85, 1.10 and 0.2 µM respectively. The results obtained in this work showed that Ajoene is as good as the control drugs.