POSTER

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BQ1 - CLONING AND CHARACTERIZATION OF DNA POLYMERASE BETA FROM *TRYPANOSOMA CRUZI*

[1]Regis-da-Silva, C.G., [2]Teixeira, S.M.R., [3]Hoffmann, J.S., [1]Pena, S.D.J., [3]Cazaux, C., [1]Machado, C.R.

[1]Laboratório de Genética-Bioquímica e [2]Laboratório de Imunologia e Biologia Celular de Parasitas-Departamento de Bioquímica e Imunologia-ICB-UFMG-Brasil; [3]Instabilité Génétique et Cancer-Institut de Pharmacologie et Biologie Structurale-France; cgustavors@yahoo.com.br,crmachad@icb.ufmg.br

The DNA polymerase beta (DNA pol b) plays a central role in base excision repair and could also interfere in other aspects of DNA repair and metabolism when overexpressed in mammalian cells. Nothing is known about the role of this enzyme and its molecular significance for the *Trypanosoma cruzi* biology. We have cloned and sequenced the *DNA pol b* from this organism. The *TcDNA pol b* sequence has 66% homology with the *Chritidia fasciculata* and *Leishmania (L.) infantum DNA pol b* genes. The *TcDNA pol b* is able to clomplement the *Escherichia coli* clone SC18-12 deficient in the *polA*$^{-}$ gene. The expression of *TcDNA pol b* in those bacterial cells restores their ability to grow at 42ºC and diminishes their sensibility to U.V. radiation. In addition, the overexpression of *TcDNA pol b* in CHO cells provokes an increase in the cells mutation rate in the 6-TG resitence assay. The TcDNA pol b protein has been expressed in *E. coli* in fusion with MBP and purified in a maltose column. The *TcDNA pol b* fusion protein was capable to add the dNTPs to primer in a DNA extension assay. Futhermore, this enzyme was used an assay of ^{3}H-dTTP incorporation measured by CPM counting to characterize its behavior in relation to dNTPs concentration. The enzyme works in optimal concentration of 5mM of dNTPs. This same assay has been used to demostrate that this enzyme has the ability to incorporate the modified nucleotide AZTTP to DNA molecules. So, we have cloned and characterized the *DNA pol b* gene from *Trypanosoma cruzi* and have started biochemical characterization of this gene product.

Supported by: CNPq, CAPES/COFECUB e PRPq-UFMG

BQ2 - THE TELOMERASE REVERSE TRANSCRIPTASE COMPONENT (TERT) OF *LEISHMANIA* SPP.: BIOCHEMICAL CHARACTERIZATION AND GENE CLONING

Giardini, M.A.[1], Fernández, M.F.[1], Lira, C.B.B.[1], Conte, F.F.[1], Camillo, L.R.[2] & Cano, M.I.N.[1].

[1] Departamento de Genética e Evolução, IB, UNICAMP, Campinas, SP; [2] Centro de Biologia Molecular e Estrutural Laboratório Nacional de Luz Síncrotron, Campinas, SP, Brazil. migiardini@yahoo.com

Telomeres are the protein-DNA complexes that protect linear chromosomes. In *Leishmania* spp. the telomeric DNA is composed by the conserved TTAGGG repeated sequence and is replicated by the action of telomerase. Telomerase is a multisubunit enzimatic complex, composed by a reverse transcriptase component (TERT), an intrinsic RNA molecule (TER) and associated proteins. It ensures the complete DNA replication by adding new telomeric sequences at the G-rich strand. The enzyme also works as part of the high order complex that regulates the capping/uncapping telomere states.

This work has two goals: i) to characterize the *in vitro* enzymatic activity of *Leishmania* telomerase and ii) to study the roles played by the TERT component in *Leishmania* life span. The main objective is to discover if telomerase can be a good target for future anti-parasite therapy.

To achieve the first goal, protein extracts of *L. (L.) amazonensis* with telomerase activity were purified using complementary chromatographic methods. Enzyme activity was tested in each purification step by using the "Two-tube TRAP" assay. The preliminary results showed that enzyme activity is found in fractions purified by anion exchange and heparin affinity chromatography. However, the activity is greatly enriched after affinity purification using a 2´-*O*-methylphosphoramidite oligoribonucleotide (Lingner & Cech, 1996). The 2´-*O*-methyl oligoprobe is complementary to the TER template sequence used by telomerase to copy the telomeric repeats, acting as a specific ligand to purify telomerase. In this case, the enzyme is eluted in native form using a displacement oligonucleotide complementary to the oligoprobe. The conventional telomerase assay is being standardized to study the enzyme catalyses features.

For the second goal, a search for sequences sharing similarities with conserved TERT domains, at the public *Leishmania* Genome Project database, resulted in a sequence from a non-assembled contig of chromosome 36 of *L. (L.) major*. A cloning strategy based on the design of primers from this putative *L. (L.) major* TERT (LmTERT) was used to amplify by PCR *L. (L.) amazonensis, L. (L.) major* and *L. (V.) braziliensis* DNA and *L. (L.) amazonensis* cDNA. PCR products of ~2.33 Kb, which correspond to two thirds of *L. (L.) major* sequence (~3.3 Kb), were amplified from all samples. The putative LaTERT, LmTERT and LbTERT share high sequence similarity, suggesting that in *Leishmania,* telomerase is a housekeeping gene. The chromosomal map of LaTERT by pulsed field gel electrophoresis, showed that it hybridized only with chromosome 36 of *L. (L.) amazonensis*, indicating that LaTERT is present in a low copy number and shows chromosome sinteny with *L. (L.) major*. RACE-PCR reactions are being standardized in order to complete the gene sequences. The genomic organization of *Leishmania* TERT and the expression of the gene during the parasite life cycle are underway.

Financial support: FAPESP, WHO/TDR – UNDP Bank.

BQ3 - CHARACTERIZATION OF A CONSTITUTIVE NITRIC OXIDE SYNTHASE FROM *LEISHMANIA (V.) BRAZILIENSIS* AND *L. (L.) CHAGASI* PROMASTIGOTES: EFFECT OF SPECIFIC INHIBITORS

Guedes-Silva, D.[1], Souza, W.J.S.[2], Leon, L.L.[1] and Genestra, M.[1]

Department of Immunology, [1] and Department of Protozoology [2] Oswaldo Cruz Institute, Oswaldo Cruz Foundation - FIOCRUZ, Rio de Janeiro, Brazil.

Nitric oxide ($NO^{\cdot}$) is a free radical derived from molecular oxygen and the guanidine nitrogen of L-arginine. NO is involved in a variety of biological functions in different cells and is an important anti-microbial effector molecule in macrophages against intra- and extra cellular pathogens. The $NO^{\cdot}$ production is catalyzed by the enzyme NO synthase (NOS), which is presented at least in two types. One (with two subtypes) is a Ca^{2+}/calmodulin-dependent that is constitutively found in the endothelium (eNOS or NOS III) and in neuronal tissue (nNOS or NOS I). The other, is inducible (iNOS or NOS II) in vascular tissues, smooth muscle cells, neutrophils, hepatocytes and macrophages, is Ca^{2+} -independent. There are few data in literature in relation to NO produced by *Leishmania* parasites, and thus, the study of NO pathway in these parasites can shown important data in reference to metabolic steps as possible target of anti-*Leishmania* drugs. A NOS was already purified from *L. (L.) donovani* and *L. (L.) amazonensis* promastigotes, but the biological significance, as well as the relationship with the macrophage pathway continues being unknown (Basu e cols., 1997: Genestra e cols., 2003a). Results of our laboratory demonstrated the NO pathway in *L. amazonensis*, that is essential for the infection establishment in murine macrophages (Genestra e cols., 2003b) and thus, in this work we decided to assay some L-arginine analogs already described as inhibitors of NOS. Parasites (*L. (V.) braziliensis*/MHOM R616 strain and *L. (L.) chagasi*/ MHOD P142) were grown in Schneider´s medium supplemented with 10% of fetal calf serum at 26°C/pH 7.2 in 24-well plates (5 x 10^5 cells/well). The group test (triplicate) was grown in the presence of the following L-arginine analogs N-nitro-L-arginine (L-NNA); N-nitro-L-arginine methyl esther (L-NAME) and D-arginine. Other alternative includes one mixture of L-NAME with L-arginine

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and/or EGTA 40 mmol/L. After incubation of 12 to 148 hours, the supernatants were used to assay the NO production by Griess reaction. The absorption was determined at a wavelength of 540 nm and the NO_2^- (a byproduct of NO) concentration in samples was determined using a standard curve of $NaNO_3$ (0,009 to 100 mmol/L) in medium. The results pointed to a significative decrease on NO production by L-NAME. Furthermore, the presence of exogenous L-arginine did not increased the NO/NO_2^- concentration, and these substrate also did not reverts the effect of L-NAME, confirming the irreversibility activity of L-NAME on NO production. Additionally, immunofluorescence assays using antibody anti cNOS demonstrated a strong immunolabeling, in comparison with a antibody anti-iNOS.

Supported by PDTIS and IOC/FIOCRUZ.

BQ4 - CHARACTERIZATION OF THE PHOSPHATIDYLSERINE SYNTHASE II CODING GENE OF *LEISHMANIA (L.) AMAZONENSIS.*

dos Santos, M. G.; Mayer, M. G.; Barcinski M. A. & Floeter-Winter, L.M.

Departamento de Parasitologia ICB USP

The life cycle of the protozoan *Leishmania* is well known. It comprehends alternative cycles in the insect and the mammalian host. However some aspects of the infection of the mammalian macrophage remain to be elucidated. It has been sugested that parasite lipid organisation can play a role in the phagocytic process and on the ability to survive in the host organism. Phosphatidylserine (PS) on the exoplasmic leaflet of the plasma membrane could be one of the signals delivered by amastigotes to inhibit the antileishmanial activity of macrophages (Balanco et al., 2001). The phosphatidylserine synthase II (PSS II) is an enzyme that exchanges the headgroup of phosphatidylethanolamine from etanolamine to serine.

The main objective of the present comunication is to isolate the complete open reading frame of PSS II from *L. (L.) amazonensis*. For that, conserved regions of the PSS II were determined by the alingment of PSS II aminoacid sequence from some phylogenetically distant organisms (*Mus musculus, Anopheles gambiae, Arabidopsis thaliana, Caenorhabditis elegans, Drosophila melanogaster, Homo sapiens* and *L. (L.) major*), obtained from GenBank. Primers were designed based on these conserved regions using the *L. (L.) major* genomic data. PCR was performed using genomic DNA from *L. (L.) amazonensis* as a substrate and the expected fragment of about 700bp was obtained.

Reverse-transcription-PCR was then performed using total RNA purified from promastigotes. The 5' and 3' regions of the PSS II coding gene were obtained, the fragments were cloned in pGEM-T easy and sequenced. The identity of the sequences in GenBank was then assured with the BLASTx program. The degrees of similarity were 37% identity and 55% positives, in relation to the human PSS II. The whole nucleotide sequence of PSS II ORF of *L. (L.) amazonensis* was then analysed to define the strategies to knock-out the gene to enable us to study its role in macrophage infection.

Financial suport: FAPESP, CNPq and CAPES

BQ5 - COMPARATIVE INHIBITORY STUDIES BETWEEN RECOMBINANT FORMS OF THE HUMAN AND *LEISHMANIA (L.) MEXICANA* GLUCOSE-6-PHOSPHATE ISOMERASE

Cordeiro, A.T.[1];Salmon, L.[2]; Delboni, F. [3]; Michels, P.A.M. [4] and Thiemann, O.T.[5]

[1,5] Laboratory of Protein Crystallography and Structural Biology, Physics Institute of São Carlos, University of São Paulo, USP, Av. Trabalhador Sãocarlense 400, PO Box 369, 13566-590, São Carlos-SP, Brazil. [2] Laboratoire de Chimie Bioorganique et Bioinorganique, CNRS-UMR 8124, Institut de Chimie Moléculaire et des Matériaux d'Orsay, Université Paris-Sud XI, 91405 Orsay Cedex, France. [3] Pontificia Universidade Católica de Minas Gerais, Campus de Poços de Caldas, Av. Padre Francis Cletus Cox, 1661, 37701-355, Poços de Caldas-MG, Brazil. [4] Research Unit for Tropical Diseases and Laboratory of Biochemistry, Christian de Duve Institute of Cellular Pathology, avenue Hippocrate 74, 1200 Brussels, Belgium. [1] artur@if.sc.usp.br; [5] thiemann@if.sc.usp.br

Glucose-6-phosphate isomerase (formerly called phosphoglucose isomerase; PGI; E.C. 5.3.1.9) is an intracellular enzyme that catalyses the reversible reaction of D-glucose 6-phosphate (G6P) to D-fructose 6-phosphate (F6P). The native *Leishmania* PGI is a homodimer molecule of 60 kDa per monomer with 47% sequence identity when compared to the human PGI. It has been shown to be present both in the cytosol and in the glycosome of *Leishmania* promastigotes. The present work describes the purification of two *Escherichia coli* expressed *L. (L.) mexicana* PGI constructs, one corresponding to the natural protein and the other to an N-terminally deleted form. The function and structure of this N-terminal segment is still unclear, but it may be related to its glycosomal localization. Four known high-energy intermediate analogue inhibitors of PGIs from *T. brucei*, *Bacillus stearothermophilus*, yeast, and/or rabbit muscle were evaluated on both recombinant human and *L. (L.) mexicana* PGIs for comparison purposes. Although the IC_{50} values obtained are 1 to 2 orders of magnitude higher than the corresponding K_i values obtained for other PGIs, probably because of the different conditions used, these compounds represent, to our knowledge, the first inhibitors ever evaluated on *L. (L.) mexicana* PGI.

Financial Support: FAPESP, CNPq and CAPES.

BQ6 - *TRYPANOSOMA CRUZI* EXPOSURE TO HYDROGEN PEROXIDE: EFFECTS ON GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND TRYPAREDOXIN PEROXIDASE

*Pereira, A. A. M., #Castilho, T., #Floeter-Winter, L., #López, J.A., @Colli, W., @Alves, M.J.M & *Gadelha, F. R.

*Departamento de Bioquímica, IB, UNICAMP, C.P. 6109, CEP13083-970, Campinas, SP, #Departamento de Parasitologia, ICB, USP, SP, @Departamento de Bioquímica, IQ, USP, São Paulo, SP, Brazil

Trypanosoma cruzi has an effective and complex system to deal with oxidative stress. Different pathways are involved in detoxifying hydroperoxides showing different sub-cellular sites and substrate specificities. In this intricate net, that converges to trypanothione, the proteins involved, cytosolic tryparedoxin peroxidase (TcCPX) included, act in concert to mediate transfer of reducing equivalents to the hydroperoxide having NADPH as the initial donor. By its turn, NADPH is produced by glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) in the pentose phosphate pathway and is involved in antioxidants pathways and reductive biosynthesis.

In the present communication the G6PD activity / expression and TcCPX expression / steady state protein level were evaluated in two *T. cruzi* strains, submitted to hydrogen peroxide (H_2O_2) treatment for different periods of time. Among other distinct biological properties, these strains have different resistance to oxidative stress generated by H_2O_2. The Y strain is less resistant to oxidative stress in contrast to the resistance showed by Tulahuen 2. Epimastigotes (5.2×10^6 cells/ml) in early stationary phase, were incubated in PBS in the presence of 50mM H_2O_2 for 30, 90 or 150 min. Cells were then collected by centrifugation and aliquots were separated for G6PD activity determination, Northern and Western blotting analysis. In Y strain, no significant changes in G6PD activity were observed except when glucose was added to the incubation medium. On the other hand, Tulahuen

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2 G6PD activity and mRNA expression could be modulated by oxidative stress. Upon 30 and 90 min incubation in the presence of the oxidant, G6PD activity increased in 40 and 60%, respectively, in relation to control. 6PGD activity remained unchanged for all times tested. mRNA levels were altered and upon 150 min incubation, an increase of 72% was observed. In the beginning of treatment no correlation could be established between G6PD and TcCPX. Western blotting analysis of TcCPX showed, after 30 min, a 38% decrease in TcCPX protein level. After 90 min mRNA levels decreased and protein expression increased. These results indicate that under oxidative stress conditions, enzymes that work in correlated pathways can be coordinately modulated. The higher G6PD activity observed in Tulahuen 2 epimastigotes and the ability of these cells to respond to H_2O_2 simultaneously with changes in TcCPx expression / protein can contribute to the higher resistance to oxidative stress and also to the higher proliferation index observed in these cells when compared to the other strain.

Supported by: FAEP-UNICAMP, FAPESP and CNPq.

BQ7 - CHARACTERIZATION OF PRENYLATED AND DOLICHYLATED PROTEINS IN *PLASMODIUM FALCIPARUM*

D´Alexandri FL, Goulart HR, Almeida IC, Peres VJ, Kimura EA e Katzin AM

Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, Av. Professor Lineu Prestes, 1374, São Paulo, SP, Brazil.

Malaria is one of the major public health problems in the world, killing over a million people each year (most of the victims are children) and infecting around 500 million (World Health Report, 2001). Therefore new drugs are urgently required for its control, since many *Plasmodium falciparum* strains have developed multi-drug resistance. Recently, it was demonstrated that the inhibition of the isoprenoid pathway (Jooma *et al*, Science 285, 1573-1576, 1999), responsible for the production of isoprenoid products, such as dolichol and isoprenic chains attached to certain proteins and coenzyme Q's isoprenic chain, could be an excellent target for the development of antimalarial drugs.

Recently Jooma *et al*. (Science 285, 1573-1576, 1999) demonstrated the presence of an isoprenoid biosynthesis pathway, the MEP pathway, in *P. falciparum* and two drugs (Fosmidomycin and FR-900098) that inhibit this pathway. Studies in our laboratory using drugs that inhibit downstream of Isopentenyl-PP (IPP) in isoprenoid pathway have been demonstrated good results, confirming this pathway as potencial target for develop new antimalarial drugs.

Other important points related about isoprenoid pathway are post-translational farnesylation, geranylgeranylation and dolichylation of protein. Prenyl modification appears necessary for the biologic activity of several proteins involved in cell cycle control, including the expanding family of *ras*-related small GTP-binding proteins (Cohen *et al*, Biochem. Pharmac., 60, 1061-1068, 2000). Our group demonstrated the existence of protein prenylation in *P. falciparum* (Moura *et al*, Antimicrob. Agents Chemoter. 45, 2553-2558, 2001) and the presence of coenzyme Q´s isoprenic chain (de Macedo *et al,* FEMS Microbiol. Lett., 207, 13-20, 2002). Couto *et al* (Biochem. J., 341, 629-637, 1999) also demonstrated the presence of dolichol of 11 and 12 isoprenic chain in the intraerythocytic forms of *P. falciparum.*

This work aimed to characterize the prenylated and dolichylated protein in the diferents intraerythocytic forms of *Plasmodium falciparum*, using the ESI-MS and QTOF mass spectrometry techniques, onto to characterized the isoprenic chain attached to this proteins.

We identified some proteins in *P. falciparum* cultures metabolic labeled with [1-(n)-^{3}H] farnesyl pyrophosphate ([^{3}H]FPP) or [1-(n)-^{3}H] geranylgeranyl pyrophosphate ([^{3}H]GGPP). When cultures were labeled with [^{3}H]FPP, we identified labeled proteins with molecular masses of approximately 14 kDa, 21-24 kDa and 50 kDa. When cultures were labelled with [^{3}H]GGPP, we identified labelled proteins with molecular masses of approximately 14 kDa and 21-24 kDa. Analysis of the isoprenic chain attached to this proteins, utilizing mass spectrometry technique (ESI-MS and QTOF), demonstrated the presence of Farnesyl group attached to proteins of 21-24 kDa and 50 kDa, Geranylgeranyl group attached to proteins of 21-24 kDa and dolichyl group attached to proteins of 21-24 kDa.

This research is sponsored by: FAPESP, CNPq, PRONEX, UNDP/World Bank/WHO

BQ8 - THE METHYLERYTHRITOL PHOSPHATE PATHWAY IS FUNCTIONALLY ACTIVE IN ALL INTRAERYTHROCYTIC STAGES OF *PLASMODIUM FALCIPARUM.*

Cassera, M. B.*; D'Alexandri, F. L.*; Peres, V. J.*; Almeida I.C.*; Gozzo F.C.‡; Kimura, E. A.* & Katzin, A. M.*

*Instituto de Ciências Biomédicas II - Departamento de Parasitologia, Av. Prof. Lineu Prestes 1374, ICB II – USP, São Paulo, SP, Brazil, CEP: 05508-900; ‡ Institute of Chemistry, State University of Campinas-UNICAMP, CP 6154, 13083-970 Campinas, SP, Brazil. bcassera@usp.br

Plasmodium falciparum synthesizes isoprenoids via a methylerythritol phosphate (MEP) pathway. Recently the first two enzymes, the PfDOXP*synthase* and PfDOXP*reductoisomerase,* were cloned and it was shown that the pesticide Fosmidomycin, a specific inhibitor of DOXP*reductoisomerase,* inhibited growth of parasite both *in vitro* and *in vivo*. In order do characterize other target enzymes of the same pathway we first demonstrated the metabolic intermediates downstream of DOXP by HPLC and Q-Tof analysis. When testing intermediates in the presence of Fosmidomycin, the levels of MEP decreased while DOXP accumulated, as expected. To our knowledge, this is the first report showing by direct biochemical detection that the MEP pathway is functionally active in all intraerythrocytic forms of *P. falciparum*. Besides, the inhibitory effect of Fosmidomycin on the isoprenoid biosynthesis (dolichols and ubiquinones) was dependent on the developmental stage: While inhibition of MEP production was observed in ring, trophozoite and schizont stages, the dolichol and ubiquinone synthesis was only affected in trophozoite and schizont stages, but not in ring stage. These results indicate that the parasite may have mechanisms to substitute isoprenoid precursors from the MEP pathway.

Supported by CNPq, FAPESP and UNDP/World Bank/WHO

BQ9 - *TRYPANOSOMA CRUZI* PROLINE RACEMASE MUTANTS : STRUCTURAL AND FUNCTIONAL STUDIES

M. Goytia[1], A. Buschiazzo[2], N. Chamond[1], F. Schaeffer[2], W.M. Degrave[3], P. Alzari[2] and P. Minoprio[1].

[1]Département d'Immunologie and [2]Département de Biologie Structurale et Chimie de l'Institut Pasteur, Paris, France; [3]Departamento de Bioquimica e Biologia Molecular, IOC, FIOCRUZ, Rio de Janeiro, Brazil.

A recently described enzyme, the first eukaryotic proline racemase, is secreted by the infective forms of *Trypanosoma cruzi*. This enzyme, *Tc*PRAC, displays a mitogenic activity towards host B cells that become polyclonally activated contributing to parasite escape and persistence inside the host. The integrity of the active site would seem critical for the mitogenic activity of the parasite released enzyme. The peptide sequence of *Tc*PRAC presents a significant homology with sequences encoding putative proline racemases in microorganisms of agricultural and medical interest. The catalytic mechanism of the proline racemase, previously only described for the protobacterium

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Clostridium sticklandii, predicted that two cysteine residues, one from each homodimer subunits, are the catalytic residues responsible for the racemization of proline enantiomers. However, the comparative analysis of peptide sequences of the *Tc*PRAC and the one of diaminopimelate epimerase monomeric enzyme (DapE), suggested that another cysteine residue upstream of the active site could as well be involved in the reaction mechanism of racemization. Using molecular, biochemical and immunological studies, we have compared in the present work wild-type *Tc*PRAC and recombinant proteins mutated for key cysteine residues located both within and outside the active site of the enzyme. We show that *Tc*PRAC possesses two functional active sites per homodimer, thus challenging the previous proposed reaction mechanism. We propose here a new enzymatic mechanism for proline racemases and present evidences that the enzymatic and mitogenic properties of *Tc*PRAC are dissociated. In this context, we show that *Tc*PRAC in presence of specific inhibitors presents a different folding that certainly prevents the correct triggering of host B cells. The identification and characterization of essential putative proline racemases in other microorganisms may allow the broad use of potential therapeutic inhibitors of *Tc*PRAC.

Support : Institut Pasteur and CNRS URA 1961.

BQ10 - ARGININE KINASE OVEREXPRESSION IMPROVES *TRYPANOSOMA CRUZI* SURVIVAL CAPABILITY

Guillermo D. Alonso[1], Soledad Ivaldi[1], Ariel M. Silber[2], León Bouvier[1]Y, Maria Júlia M. Alves[2], Héctor N. Torres[1], Mirtha M. Flawiá[1] and Claudio A. Pereira[1,*].

[1]Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, Universidad de Buenos Aires, Argentina. [2]Laboratório de Bioquímica de Parasitas, Instituto de Química, Universidade de São Paulo, São Paulo, Brazil. * Email: cpereira@dna.uba.ar

Arginine kinase catalyzes the reversible transphosphorylation between phosphoarginine and ADP. Phosphoarginine is a metabolite that is involved in temporal and spatial ATP buffering as well as inorganic phosphate regulation. Recently, we demonstrated that arginine kinase is present in the parasites *Trypanosoma cruzi* and *Trypanosoma brucei*. In turn, this enzyme is absent in their mammalian hosts. In this work we establish a relationship among the homologous overexpression of the *Trypanosoma cruzi* arginine kinase and the ability of the transfectant parasite cells to grow and resist stress conditions. Using the novel expression vector pTREX it was obtained more than one hundred-fold overexpression of the *T. cruzi* arginine kinase activity. The stable-transfected parasites showed an increased cell density since day 10 of culture, which resulted about 2.5-fold higher than the control group on day 28. These results suggest an improved capability of the transfected parasites to survive in a nutrient-depleted medium. Additional stress conditions were tested by incubating the parasites in different media. Arginine kinase transfected parasites revealed about 54% of increase in the cell number growing in Triatomine Artificial Urine medium, 91% in alkaline PBS and 79% in conditioned medium. We propose that arginine kinase and phosphoarginine are involved in the regulation of the *Trypanosoma cruzi* growth and its adaptation to environmental changes.

Supports: Third World Academy of Sciences, Agencia Nacional de Promoción Científica y Tecnológica, University of Buenos Aires, Fundación Antorchas and Consejo Nacional de Investigaciones Científicas y Técnicas.

BQ11 - MOLECULAR CLONING AND EXPRESSION OF THE *TRYPANOSOMA CRUZI* METHYLTHIOADENOSINE PHOSPHORYLASE GENE.

Neves, D[1]., Noleto, C[1]., Grellier, P[3]., Engman, D.[2], Teixeira, A.R.L.[1], and Santana, J.M.[1]

[1]Laboratório Multidisciplinar de Pesquisa em Doença de Chagas, Universidade de Brasília, Brasília-DF; corresponding author: jsantana@unb.br [2]Northwestern University Medical School, Chicago [3]Museum Nationalle d´Histoire Naturelle, Paris

Trypanosomatids have an absolute requirement for purines because they lack the machinery to synthesize their own purine ring, *de novo* synthesis. Purines serve as precursor molecules for DNA and RNA, as carriers of high-energy phosphate bonds, as constituents of coenzymes and as modulators of certain enzymes. Purines are found and available to these parasites as nucleobases, nucleosides, charged nucleotides, or polymers in nucleic acids. The methylthioadenosine (MTA), a byproduct from the polyamines production, is cleaved by a specific MTA phosphorylase (MTAP) into adenine and methyl-thioribose-1-phosphate (MTR1P). Adenine is converted to adenine nucleotides (purine salvage pathway) and MTR1P intermediate can be recycled into methionine. This pathway is potentially exploitable for chemotherapy target in protozoan parasites because of the needs of pre-formed purines. The MTAP from *Trypanosoma cruzi* has been poorly studied and needs better characterization to evaluate the possibility of its use as a drug target. Our objective is to characterize molecularly and functionally this enzyme. For this, we identified its gene that shows high identity with MTAPs from different organisms. It is present as a single copy per haploid genome of the parasite and expressed in all of its forms. To obtain active rMTAP successfully in *E. coli*, the full-length proteinase gene ORF was inserted into the pET-19b expression vector to generate an N-terminal His-tagged recombinant protein. The recombinant protein was then purified from soluble and insoluble fractions utilizing a column charged with nickel. The recombinant MTAP showed approximately a 33 kDa protein in SDS-PAGE both reducing or non-reducing conditions. The antibodies raised against the recombinant protein specifically recognized the native MTAP in immunoblots with total protein extract from trypomastigote, epimastigote or amastigote forms of *T. cruzi*. The rMTAP from the soluble fraction was shown to be fully active on MTAP substrate. The assay of rMTAP activity was done coupling the enzymatic reaction with xanthine oxidase, which converts free adenine to 2,8-dihydroxyadenine. The expression of an active recombinant allows a better inhibitor screening and the production of MTAP crystals to determine the three-dimensional structure. The complete *T. cruzi* MTAP characterization can elucidate its relevance to the metabolism of the parasite as well as the evaluation of its potential as a drug target.

This research is supported by CNPq.

BQ12 - CHARACTERIZATION OF ECTO-ENZYMES IN SHORT AND LONG EPIMASTIGOTES OF *TRYPANOSOMA RANGELI.*

Gomes, S.A.O.; Fonseca de Souza, A. L.; Meyer-Fernandes, J.R.

Laboratory of Cellular Biochemistry - Department of Medical Biochemistry-Federal University of Rio de Janeiro-UFRJ.

Trypanosoma rangeli is transmitted through triatominae vectors, during a blood meal on man and other mammals. It develops predominantly as short epimastigotes forms in the gut of the insect, invades the haemocoel and a few days after infection they disappear to be replaced by a massive colonization by long epimastigotes forms. The long epimastigotes survive in the haemolymph and/or to get into the haemocytes, migrate to and complete their development in the salivary glands where the metacyclogenesis (trypomastigote formation) takes place. Data already published, demonstrated a higher defense reaction when short, but not long epimastigotes of *T. rangeli* were inoculated into the haemolymph of the insect vector, *Rhodnius prolixus*.

Surface membrane interactions between parasites and their host cells are of critical importance for the survival of the parasite. The plasma membranes of cells contain enzymes whose active sites face the external medium rather than

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cytoplasm. The activities of these enzymes, referred as ecto-enzymes, can be measured using living cells. The regulation of the complex interactions required for trypanosomatidae differentiation and proliferation is mediated in part by protein phosphorylation. In several protozoa parasites ecto-phosphatase have been described, although the physiological role has not been well established, it seems to be involved with nutrition and cell differentiation. Many enzymes working together are responsible to the phosphate transport. Phosphatases and ATPases are examples of these enzymes. The ATP hydrolysis offers energy for to get phosphate into the cells.

In the present study we described experiments about the development of short and long epimastigotes at different phosphate concentrations in LIT medium and also analized ecto-phosphatase and ecto-ATPase activities on the surface of *T. rangeli*. The observed results were: (i) short epimastigotes requires higher phosphate concentrations than long epimastigotes; (ii) both ecto-phosphatase activities Mg^{2+}dependent and independent from short epimastigotes dephosphorylated more efficiently *p*-nitrophenylphosphate (*p*-NPP) as well as b-glycerophosphate than long epimastigotes; (iii) in addition, ecto-ATPase activity of short epimastigotes was greather than long epimastigotes; (iv) the inorganic phosphate determination in the haemolymph of *Rhodnius prolixus* showed a low concentration of this compound. These findings will be discussed on the light of the knowledge of *T. rangeli* development in the triatominae vector.

This work was supported by CNPq and FAPERJ.

BQ13 - CHARACTERIZATION AND IMMUNOLOCALIZATION OF AN ATP-DIPHOSPHOHYDROLASE OF *TRYPANOSOMA CRUZI* AND ITS POSSIBLE ROLE IN VIRULENCE PROCESS

Juliana L. R. Fietto [c], Ricardo DeMarco [a], Ivan P. Nascimento[a], Ieso M. Castro [c] Técia M. U. Carvalho[b], Wanderley de Souza[b], Maria T. Bahia [c], Maria J. M. Alves[a] and Sergio Verjovski-Almeida[a]

[a]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, Caixa Postal 26077, 05599-970 São Paulo, SP, Brazil; [b]Universidade Estadual do Norte Fluminense, 28015-620 Campos dos Goytacazes, RJ, Brazil and [c] Núcleo de Pesquisas em Biologia, Universidade Federal de Ouro Preto, MG, Brazil.

In this work an ecto-NTPDase insensitive to inhibitors of other ATPases and phosphatases has been characterized on the surface of *Trypanosoma cruzi* intact parasites (strains Y and Be-78). The enzyme exhibits a broad substrate specificity for nucleotide hydrolysis, typical for the family of ATP-diphosphohydrolases, which are known to be present on the surface of other endoparasites such as *Schistosoma mansoni*, *Toxoplasma gondii* and *Leismania amazonensis*. Antibodies against a fusion protein from *T. gondii* ATP-diphosphohydrolase immunoprecipitated a single 58 kDa protein from ^{35}S-methionine labeled *T. cruzi* parasites, confirming that *T. cruzi* enzyme possesses epitopes which are common to the family of ATP-diphosphohydrolases. Epimastigotes (non-infective form) and tripomastigotes (infective form) from strain Y showed different ATP/ADP hydrolysis, infective form showed a preference for ATP and non-infective form hydrolyzed both nucleotides at the same rate. Confocal fluorescence microscopy analyses localized the NTPDase on the external surface of all forms of parasite, with trypomastigotes and amastigotes having higher contents of ATP-diphosphohydrolase than epimastigotes and metacyclic forms. The NTPDase on the surface of *T. cruzi* could be part of the *T. cruzi* purine salvage pathway and also might play a role in the escape mechanisms of the parasite by degrading ATP, ADP or other nucleotides eventually involved in different process such as inflammation and modulation of immune responses.

Financial support: CNPq, FAPESP, UFOP

BQ14 - PURIFICATION OF AN ECTO-ATPASE INVOLVED IN ADENOSINE ACQUISITION IN *LEISHMANIA (L.) AMAZONENSIS* PROMASTIGOTE.

MARTINS-DUARTE, E.S., PINHEIRO, C.M., FERRARO, R.B., MEYER-FERNANDES, J.R.

Departamento de Bioquímica Médica, ICB, UFRJ, Rio de Janeiro, Brasil

Leishmania spp., as others parasites of Trypanosomatidae family, is unable to synthesize purines *de novo*, requiring exogens purines for its growth. The metabolism and purines transport across parasites plasma membrane represent a promising target for diseases treatment caused by trypanosomatides. The plasma membrane of these cells contains enzymes whose active sites face the external medium rather than the cytoplasm. These enzymes are referred to as ecto-enzymes and their activities can be measured using living cells.

The capacity of *Leishmania (L.) amazonensis* intact promastigotes hydrolyzes nucleotides was analyzed by isocratic ion-pair reversed-phase HPLC. The parasites were incubated with ATP 100 mM, aliquots were took in variable times and analyzed in HPLC in order to determine concentration of ADP, AMP and adenosine, as well as, the reminiscent ATP present in the medium. It was verified that ATP concentration decreased rapidly with subsequent generation of ADP, AMP and adenosine. Initially the ADP concentration increased, but some minutes later it began to decrease due to surface ADPase activity of *L. (L.) amazonensis*.

A plasma membrane fraction was obtained and the enrichment was verified measuring 3'nucleotidase activity. The solubilized plasma membrane proteins was applied in poliacrilamida gel under nondenaturing conditions and stained for enzyme activity. The band that exhibited ATPase activity was excised, analysed by SDS-PAGE and stained with silver.

More studies are being performed for a better understanding of enzyme structure and functions, such as the relation with other ecto-ATPases and apyrases already described on literature.

Supported by: CNPq, PRONEX, FAPERJ, FINEP e CAPES.

BQ15 - AN ECTO-ATPASE IN *ENTAMOEBA HISTOLYTICA* AND ITS POSSIBLE INVOLVIMENT WITH VIRULENCE

Kiffer, T., Barros, F. S., Silva-Filho, F. C., Rocha-Azevedo, B., Meyer-Fernandes, J. R., Pinheiro, A. A. S.

Entamoeba histolytica is an enteric protozoa with high pathogen potential for human being due to its ability to disrupt and invade the colonic mucosa. The pathophysiology of amoebiasis is a multifactorial process which includes adhesion and colonization of the intestinal mucosa, damage in epithelial layer, lysis of inflammatory cells of host immune defense followed by penetration in other tissues.

During the last two decades, considerable progress has been achieved in the study of ecto-nucleotidases in general and ecto-ATPases in particular. Ecto-ATPases are glycoproteins present in the plasma membrane and have the active site facing the external medium rather than the cytoplasm. In different protozoa including some members of *Trypanosomatidae* family, it has been described the presence of ecto-ATPases able to hydrolyse extracellular ATP. Our group characterized a Mg^{2+}-dependent ecto-ATP diphosphohydrolase activity present in the surface of *E. histolytica* which is inhibited by DIDS and Suramin and stimulated by galactose, one of the recognized sugar by the surface lectin Gal/GalNAc of amoeba. In this work, the possible involvement of this activity in parasite-host cell interaction was evaluated by the influence of different modulators of this enzymatic activity in the cytotoxic effect of this parasite under HeLa cells. In these interaction experiments, epithelial cells were previously prepared and incubated with [^{3}H]Timidine, so that the lysis of monolayer can be measured by counting the radioactivity in coculture supernatant by liquid cintillation. After 30 minutes of

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interaction, the time course was linear with time at least four hours of incubation and with cell density until the ratio of 3:1 (trophozoite:HeLa cells) achieving 40% of lysis of the monolayer. To test the efficiency of the interaction system used in this work to measure citotoxicity, the effect of galactose was tested. This result shows that the addition of galactose (50 mM) in the interaction medium reduced the lysis of HeLa cells in 40%. Similar results were observed when the monolayer was pretreated with PCLEC (10 µg/mL) and TEL (10 µg/mL), two different lectins specific to galactose and GalNAC, respectively. The effect of ATP, preferential substrate to *E. histolytica* ecto-ATPases, was analysed. This nucleotide added to the interaction medium reduced in 40% the citotoxic effect of the parasite. The same protector effect was observed to DIDS (1 mM), the inhibitor agent of the ecto-ATPase activities observed in E. histolytica. When DIDS and galactose were added concomitantly in the reaction mixture the protective effect was enhanced. These results suggest a possible role of ecto-ATPases in the interaction process between *E. histolytica* and host cell.

Supported by: CNPq, FAPERJ, CAPES and PRONEX.

BQ16 - CHARACTERIZATION OF AN ECTO-PHOSPHATASE ACTIVITY PRESENT IN EPIMASTIGOTES OF *TRYPANOSOMA RANGELI* AND ITS POSSIBLE ROLE IN NUTRIENTS ACQUISITION

Fonseca de Souza, A.L.; Vasconcelos, F.F.; Meyer-Fernandes, J.R.

Laboratory of Cellular Biochemistry - Department of Medical Biochemistry-Federal University of Rio de Janeiro-UFRJ.

Trypanosoma rangeli is a digenetic hemoflagelated parasite widely distributed on the Central and South Americas. It's able to infect several animal groups, as well as humans. The life cycle begins with the ingestion of trypomastigote forms present in vertebrate bloodstream by the triatominae. Inside of the vector gut, parasites differentiate to epimastigote forms and pass through the gut epithelium, achieving the hemocoel. Parasites proliferate in the haemolymph or into the haemocytes and migrate to the salivary glands where trypomastigote is formed. This stage is able to infect the vertebrate host during new blood feed.

In protozoa parasites, surface protein phosphorylation and dephosphorylation events are involved with host-cell interaction, cell differentiation, secretion of toxic factors and nutrients acquisition. Thus, the detection of kinases and phosphatases activities on the parasaites surface is of great relevance to the comprehension of its biochemistry.

This work proposes the characterization of an ecto-phosphatase activity present in *T. rangeli* and its possible involvement in nutrients acquisition. Living cells of *T. rangeli* are able to hydrolyze the artificial substrate *p*-nitrophenylphosphate (*p*-NPP), linearly with time and with cell density. *p*-NPP hydrolysis presented a Michaelian kinetic with apparent K_m and V_{max} values of 2.6 ± 0.26 mM and 6.62 ± 0.17 nmol *p*-NP / h x 10^7 cells, respectively. $MgCl_2$ and $CuCl_2$ stimulated the phosphatase activity and Mg^{+2} stimulation was dose-dependent. Levamizole and tartrate, alkaline and secreted phosphatase inhibitors, respectively, were not able to modulate this activity. However, NaF, molybdate and vanadate, three classic acid phosphatase inhibitors, decreased considerably the control activity. This ecto-phosphatase activity was also able to dephosphorylate phosphoaminoacids. Phosphoserine hydrolysis was stimulated by Mg^{2+} as well as *p*-NPP hydrolysis, suggesting the presence of more than one enzyme on the parasite surface with different specificities. Our findings demonstrated that, at low phosphate concentrations, the epimastigote cells of *T. rangeli* did not grow efficiently. Nevertheless, when β-glycerophosphate, a usual substrate for phosphatase activities, was added in the culture medium the growth rate became higher. These observations suggest that this phosphatase activity present on the surface of *T. rangeli* may participate to the mechanisms of nutrients acquisition in this trypanosomatid.

This work was supported by CNPq and FAPERJ.

BQ17 - PRESENCE OF ACIDOCALCISOME IN *LEPTOMONAS WALLACEI*.

Medeiros, L.C.A.S.[1], Monteiro, C.S.[1], Miranda, K.[2], Moreira, B.L.M.[1], De Souza, W.[2] & Barrabin, H.[1]

1- Laboratório de Membranas Transportadoras, Departamento de Bioquímica Médica, ICB, CCS, UFRJ. 2- Laboratório de Ultraestrutura Celular Hertha Meyer, IBCCF, CCS, UFRJ.

Acidocalcisomes are acidic calcium-storage organelles found in several microorganisms. They are characterized by their acidic nature, high electron density, high content of polyphosphates, and a number of pumps and exchangers responsible for the ion uptake into these organelles. In this work, we show that *Leptomonas wallacei*, a trypanosomatid isolated from the phytophagous insect *Oncopeltus fasciatus*, possess a electron dense organelles with structural, chemical and physiological properties similar to the acidocalcisomes. Pyrophosphate-driven H^+ uptake was measured in cells permeabilized by digitonin using acridine orange, a dye that changes its absorbance when accumulated in acidic compartments, as a probe. The H^+-pyrophosphatase activity was inhibited by sodium fluoride (NaF), imidodiphosphate (IDP), H^+-pyrophosphatases inhibitors. H^+ was released with the addition of 250µM Ca^{2+}, suggesting the presence of a Ca^{2+}/ H^+ antiport in internal compartments. However, Na^+ was unable to release protons from these organelles. The H^+-pyrophosphatase activity was optimal in the pH range of 7.0 to 7.5. This activity was completely dependent of ion K^+ and independent of Na^+ and sucrose. The maximal activity occurred with 130 mM of KCl. The pyrophosphate-driven proton uptake was dependent on the PPi concentration. However, due to the fast substrate consumption, we were not able to obtain a value for half-maximal activation. Maximal values for H^+ transport were obtained at concentrations of PPi above 50 µM. In addition, X-ray elemental mapping associated with energy-filtering transmission electron microscopy showed that most of the cations, namely Na, Mg, P, K, Fe and Zn are located in the acidocalcisome matrix.

These results suggest that *Leptomonas wallacei* possess an organelle that is able to accumulate H^+ using the energy coupled from pyrophosphate hydrolysis. This organelle possess a Ca^{2+}/ H^+ antiport and a H^+-pyrophosphatase which is inhibited by IDP and NaF, as described in acidocalcisomes. In addition, electron-dense organelles with structural properties and elemental composition similar to the acidocalcisomes were identified. However, in contrast to the other trypanosomatids so far studied, we did not identify the presence of a H^+-ATPase sensitive to bafilomycin A (V-H^+-ATPase) neither a Na^+/ H^+ antiport in *Leptomonas wallacei*.

Support by CNPQ, PRONEX e FAPERJ.

BQ18 - INTERACTION BETWEEN PLATELET ACTIVATING FACTOR (PAF) AND *TRYPANOSOMA CRUZI*: BIOLOGICAL ACTION AND MOLECULAR ASPECTS OF A POSSIBLE RECEPTOR.

Ambrosio, L.[1] Aliberti, JC[2]; Molfetta JB[3]; Guaratini T[1]; Lopes NP[1]; Massuo J. Kato[4]; Tedesco AC[3]; Yamauchi, LLM[4]; Silva JS[4] and Dias-Baruffi, M[1].

Faculdade de Ciências Farmacêuticas de Ribeirão Preto-USP[1]; National Institute of Health- USA[2]. Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto-USP[3], Faculdade de Medicina de Ribeirão Preto-USP[4].

Chagas disease is caused by the protozoan *Trypanosoma cruzi* (*T. cruzi*). It represents one of the main public health concerns in Latin America. Data from the literature have suggested the presence of a platelet activating factor receptor

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(PAF) in *T. cruzi.* This lipid mediator induces, by direct action on the parasite, enzyme secretion, cell differentiation, and signal transduction. The goals of this work were to study some biological effects of PAF on *T. cruzi* and to obtain a preliminary molecular analysis of a possible receptor gene for this lipid mediator in *T. cruzi*. Trypomastigote suspensions were treated with PAF and the resulting biological effects were monitored by *in vitro* morphological transformation assays, evaluation of parasite migration to artificial cavities in mice pre-infected with this parasite, as well as spectrofluorimetric assays to quantify calcium influx. Results from these experiments show that PAF (10^{-6}M) induces transformation of 30% of the trypomastigote forms into amastigote-like forms, after incubation for 120 minutes. Interestingly, this effect was blocked by pertussis toxin or Veragensin in approximately 50% of the parasites. Moreover, PAF induced parasite migration to the dorsal air pouch in mice, in a dose-dependent manner, with the most effective dose being 10^{-6}M. This mediator was also able to induce calcium influx in trypomastigotes. This influx was affected by different doses and incubation times. In order to investigate the presence of a PAF receptor gene in *T. cruzi*, polymerase chain reaction (PCR) assays were performed using cDNA and/or genomic DNA from different forms of *T. cruzi*. Oligonucelotides, homologous to the PAF receptor gene from mammals, were utilized in these reactions. It was possible to amplify DNA fragments (350 and 1450 base pairs) with these oligonucleotides from trypomastigote cDNA. Based on the results obtained, we suggest that PAF may modulate some biological events in *T.cruzi* infection by interacting with a possible G protein-coupled receptor. These findings may add some insights to studies on the interaction of *T.cruzi* with the vertebrate host immune defense system and on the physiopathology of Chagas disease.

Financial Support: FAPESP – CAPES - CNPq

BQ19 - INVOLVEMENT OF CYCLIC AMP AND PROTEIN KINASE A IN CELL DIFFERENTIATION TRIGGERED BY PLATELET-ACTIVATING FACTOR IN *HERPETOMONAS MUSCARUM MUSCARUM*

Danielle P. Vieira*, Fernando L. Dutra*, Mário A. C. Silva Neto** and Angela H. C. S. Lopes*

*Instituto de Microbiologia Prof. Paulo de Góes, UFRJ; **Departamento de Bioquímica Médica, ICB, UFRJ; Cidade Universitária, Ilha do Fundão, Rio de Janeiro, R.J. 21941-590, Brasil. E-mail: angela.lopes@micro.ufrj.br

Herpetomonas muscarum muscarum is a house fly flagellate parasite of the family Trypanosomatidae that presents three evolutive forms: promastigote, paramastigote and opisthomastigote. These parasites have been widely used as a model for cell biology and biochemistry of lower as well as higher eukaryonts. These trypanosomatids resemble higher eukaryonts in several aspects, including the fact that their cellular functions are mediated by signaling pathways involving protein kinases and phosphatases, G proteins and second messengers. Cyclic AMP (cAMP) is an important second messenger that regulates functions such as cell proliferation, differentiation and host cell invasion by parasites. Most of the effects induced by cAMP are mediated through protein kinase A (PKA), a protein kinase dependent on cAMP, whose targets have not been identified in parasites yet. Platelet-activating factor (PAF) is a potent phospholipid mediator of several cellular functions in diverse biological and pathophysiological processes, such as cell differentiation, inflammation and allergy. In earlier studies, we have demonstrated that PAF triggers the process of cell differentiation in *H. m. muscarum* and in *Trypanosoma cruzi.* Recently we showed that PAF triggers a cascade of signal transduction events, leading to the activation of casein kinase II (CKII), through protein kinase C (PKC) in *H. m. muscarum.* In this work, the concentration of intracellular cAMP was assayed in the cytoplasmic content of *H. m. muscarum* grown in the absence or in the presence of PAF (10^{-7}M), showing that this lipid mediator promoted a two-fold increase in the concentration of cAMP. Taking this result into account, we decided to study the effect of cAMP on cell differentiation of *H. m. muscarum*. The parasites were grown for periods ranging from 1 to 3 days, in the absence or in the presence of the following drugs: PAF (10^{-7}M) and/or cAMP (10mM) and/or the PKA inhibitor H89 (10mM). The percentage of non-differentiated forms (promastigotes) and differentiated forms (paramastigotes plus opisthomastigotes) was daily determined by using Giemsa stained preparations. On the third day of incubation, parasites grown in the presence of these modulators presented the following percentage of differentiated forms: PAF (70%), cAMP (75%), H89 (42%), PAF + cAMP (75%), PAF + cAMP + H89 (43%), cAMP + H89 (38%), PAF + H89 (32%), as compared to the control parasites, which presented 40% differentiated forms. Together, this set of results suggests that PAF and cAMP stimulate cell differentiation in *H. m. muscarum* in a PKA activity-dependent fashion.

Supported by: CNPq, FAPERJ, CNPq/PIBIC-UFRJ and PRONEX (0885).

BQ20 - PLATELET-ACTIVATING FACTOR-TRIGGERED CELL DIFFERENTIATION IN *TRYPANOSOMA CRUZI* IS DEPENDENT ON CYCLIC AMP AND PROTEIN KINASE A

Marta Teixeira Gomes*, Danielle P. Vieira*, Mário A. C. Silva Neto** and Angela H. C. S. Lopes*

*Instituto de Microbiologia Prof. Paulo de Góes, UFRJ; **Departamento de Bioquímica Médica, ICB, UFRJ; Cidade Universitária, Ilha do Fundão, Rio de Janeiro, R.J. 21941-590, Brasil. E-mail: angela.lopes@micro.ufrj.br

Trypanosoma cruzi, a protozoan parasite that exhibits developmental regulation of virulence, is transmitted by reduviid insects. These insects become infected by ingesting trypomastigotes from the blood of the mammalian hosts; the parasites then multiply as epimastigotes and differentiate into metacyclic trypomastigotes in the lumen of the crop and midgut. Mechanisms underlying the differentiation of the parasite are poorly understood, although we do know that platelet-activating factor (PAF) triggers the differentiation of *T. cruzi* from epimastigotes into trypomastigotes. PAF is a potent phospholipid mediator of several cellular functions in diverse biological and pathophysiological processes, such as cell differentiation, inflammation and allergy. *T. cruzi*, like other eukaryonts, present cellular functions mediated by signaling pathways involving protein kinases and phosphatases, G proteins and second messengers. Cyclic AMP (cAMP) is an important second messenger that regulates functions such as cell proliferation, differentiation and host cell invasion by parasites. Most of the effects induced by cAMP are mediated through protein kinase A (PKA), a protein kinase dependent on cAMP, whose targets have not been identified in parasites yet. Recently we showed that PAF triggers a cascade of signal transduction events, leading to the activation of casein kinase II (CKII), through protein kinase C (PKC) in the trypanosomatid *Herpetomonas muscarum muscarum.* In this work we studied the effect of cAMP on cell differentiation of *T. cruzi*, clone Dm28c *in vitro*. The parasites were maintained in TAUP medium for periods ranging from 1 to 6 days, in the absence or in the presence of the following drugs: PAF(10^{-6}M) and/or cAMP (10mM) and/or the PKA inhibitor H89 (10mM). The percentage of epimastigotes and trypomastigotes was daily determined by using Giemsa stained preparations. On the third day of incubation, parasites maintained in TAUP medium in the presence of these modulators presented the following percentage of trypomastigotes: PAF (81%), cAMP (76%), H89 (35%), PAF + cAMP (82%), PAF + cAMP + H89 (43%), cAMP + H89 (45%), PAF + H89 (52%), as compared to the control parasites, which presented 48% trypomastigotes. Together, this set of results suggests that PAF and cAMP stimulate cell differentiation in *T. cruzi*, which is inhibited by the PKA inhibitor H89. Taking this result into account, we decided to study the effects of these modulators on the protein profile of *T. cruzi* extracts by SDS PAGE. Accordingly, it was observed that the protein extract from *T. cruzi* parasites maintained in TAUP medium for one hour in the presence of 10^{-6} M PAF or cAMP presented a

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major difference in the amount of a protein of 60 kDa and of both this protein and another one of 105 kDa in PAF or cAMP-treated parasites, respectively, as compared to the control flagellates. This phenomenon was suppressed by H89, which correlates well with the cell differentiation results.

Supported by: CNPq, FAPERJ, CNPq/PIBIC-UFRJ and PRONEX (0885).

BQ21 - CASEIN KINASE 2 (CK2) AND PROTEIN KINASE C (PKC) ACTIVITIES IN *LEISHMANIA (V.) BRAZILIENSIS*: A COMPARATIVE STUDY BETWEEN INFECTIVE AND NON-INFECTIVE STRAINS

Brito, A.M.[1], Souza, J.E.O.[1], Lopes, A.H.C.S.[2], Silva-Neto, M.A.C.[3] & Dutra, P.M.L.[1]

[1]Disciplina de Parasitologia, DPL, FCM, UERJ. [2]Instituto de Microbiologia Prof. Paulo de Góes, UFRJ; [3]Departamento de Bioquímica Médica, ICB, UFRJ; Cidade Universitária, Ilha do Fundão, Rio de Janeiro, R.J. 21941-590, Brasil. E-mail: pdutra@uerj.br.

Parasites of the genus *Leishmania* are transmitted by the bite of sand flies and infect cells of the mononuclear phagocyte lineage of their vertebrate hosts. Depending both on the virulence factors of the parasite itself and on the immune response established by the host, a spectrum of diseases known as leishmaniasis can appear, and these can be cutaneous and/or visceral. Approximately 350 million people live in areas of active transmission of *Leishmani*a, with 12 million people throughout Africa, Asia, Europe, and the Americas directly affected by leishmaniasis. The metacyclogenesis process (acquiring of infectivity), is very important to the *Leishmania*-macrophage interaction. This process may modify the molecules of the parasite surface and, by thus, promoting the activation of signal transduction pathways. The study of the enzymes related to phosphorylation and dephosphorylation of proteins present on the external surface of these parasites is of fundamental importance. Several ecto-enzymes have been described in the trypanosomatids, including ecto-phosphatases and ecto-kinases. Casein kinase 2 activities have been described both on the cell surface and as secreted enzymes of *Leshmania (L.) major* and *L. (L.) amazonensis*. These enzymes seem to be involved with cell growth, differentiation and infectivity. In the present work, we have identified two kinase activities (CK 2 and PKC) in two strains of *Leishmania braziliensis*: an infective and non-infective one. These enzymes are present on external cell membrane of these parasites, in the cytoplasm and as a secreted form. The *Leishmania (V.) braziliensis* infective strain (MHOM/BR/2002/EMM-IOC-L2535) was recently isolated from a patient. This strain was inoculated in hamster footpad and recovered 9 weeks after infection. The *Leishmania (V.) braziliensis* non-infective strain (CT-IOC-238-L566) has been kept axenically in culture for several years. The ecto-CK2, the intracellular CK2 and the secreted CK2 activities were much higher in the infective strain than in the non-infective one. However, the enhancement induced by the addition of the substrate casein (1mg/mL) on the secreted CK2 activity was more pronounced in the non-infective strain (4-fold). The addition of casein promoted 37% inhibition of the CK2 activity present on the external surface of *L. (V.) braziliensis* infective parasites. The PKC activity presented an opposite pattern, as it was 30-fold higher in the non-infective strain than in the infective one.

Supported by: Supported by: TWAS (RGA No. 01-110 RG/BIO/LA), CNPq, FAPERJ, CNPq/PIBIC-UERJ and PRONEX (0885).

BQ22 - ENDOSYMBIOSIS IN TRYPANOSOMATIDS: THE SYMBIOTIC BACTERIUM INFLUENCES THE POLYAMINE METABOLISM IN THE HOST PROTOZOAN

Frossard, M.L[1], Seabra, S.H.[1], Da Matta, R.A.[2], De Souza, W[1]. Motta, M.C.M.[1] and Mello F.G.[3]

1. Laboratório de Ultraestrutura Celular Hertha Meyer, UFRJ; 2. Laboratório de Biologia Celular e Tecidual, UENF; 3. Laboratório de Neuroquímica, UFRJ. seabra@biof.ufrj.br

Some protozoa of the Trypanosomatidae family presents an endosymbiotic bacterium which is enclosed by two unit membranes and is usually found close to the host cell nucleus. A cured strain of such species can be obtained after antibiotic treatment, allowing a better understanding of this symbiotic association. The endosymbiont furnishes essential nutrients to the host cell, resulting in higher proliferation capacity of the endosymbiont-bearing trypanosomatid when compared to cured strains. The ornithine decarboxylase (ODC) is involved in polyamine biosynthesis and its activity is related to the cell division capacity. Western blotting analysis showed that both strains have similar amounts of ODC, while endosymbionts obtained after cell fractioning did not present such enzyme. However, the ODC activity is higher in endosymbiont-bearing trypanosomatids when compared to the results obtained for the cured strain. In order to better understand the differential ODC activity, the cured strain of *Crithidia deanei* was grown in conditioned culture medium, which was obtained after 24 h of cultured endosymbiont-bearing strain. The results showed an increase in cell proliferation and in the ODC activity, when the cured strain was cultivated in conditioned medium or after addition of endosymbiont extract to the culture medium. Taken together, these data suggest that the endosymbiont can enhance the protozoan ODC activity by providing factors that increase the polyamine metabolism. Regarding the ODC localization, the endosymbiont-bearing strain displayed immunolabeling in the cytoplasm and in the flagellar pocket after incubation with a polyclonal antibody anti-ODC. The cured strain showed a different ODC localization, since immunofluorescence was only observed over the cytoplasm. The cultivation of the aposymbiotic strain in the conditioned medium resulted in a new distribution of the enzyme, since the flagellar pocket was also labeled, as in the normal strain. These results suggest that the ODC activity is important for *C.deanei* growth, which is dependent on factors eventually produced by the endosymbiont.

Supported by: CNPq, FAPERJ and FUJB.

BQ23 - THE USE OF AN ALKYL-LISOPHOSPHOLIPID TO STUDY THE SYMBIOTIC RELATIONSHIP IN TRYPANOSOMATIDS

Ferreira, C.F.B.[1]., Frossard, M.L.[1], Cavalcanti, D.P.[1], De souza, W.[1], Einicker-Lamas, M.[2] and Motta, M.C.M[1]

1 - Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, UFRJ, Rio de Janeiro, RJ, Brazil; 2 - Laboratório de fisico-Química Biológica, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, UFRJ, Rio de Janeiro, RJ, Brazil

Alkyl-lysophospholipids (ALP), originally developed as anticancer drugs, have shown a significant antiproliferative activity against trypanosomatids. The effect of these compounds have been related to perturbation of the alkyl-lipid metabolism and to the biosynthesis of phospholipids, as well as damage to celullar membranes. In this study we verified the effects of ET-18-OCH3, an ALP, in *Crithidia deanei*, an endosymbiont-harbouring trypanosomatid. There is a controversy involving the origin of the endosymbiont envelope; it can be derived from the host trypanosomatid or it can presents prokaryotic features. Thus, the lipid composition of this symbiotic bacterium has been investigated in order to elucidate this question. Phosphatidycholine (PC), is the major membrane phospholipid in eukaryotes, however it is found in only a few species of bacteria, including the symbiotic ones. Enzymatic methylation of phosphatidylethanolamine (PE) is the main biosynthetic pathway to yield PC in bacteria. However, in symbiotic associations this phospholipid can be directly

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synthesized from choline produced by the host cell. Taken together, these data suggest that PC may be required for a successful interaction of the symbiont with the host. Previous studies revealed that the major phospholipid in the endosymbiont of *C. deanei* is phosphatydilcoline, followed by cardiolipin, phosphatidylinositol and phosphatidylethanolamine. Recent data showed that the ET-18-OCH3 had a dose-dependent effect on cell proliferation and also promoted ultrastructural modifications in *C. deanei*. Ultrastructural analysis by transmission electron microscopy showed plasma membrane shedding, mitochondrion swelling and damage of the endosymbiont envelope. Recent biochemical analysis showed that such morphological alterations are related to the drug effect on phospholipid biosynthesis. Now, we are verifying the effect of ET-18-OCH3 on the phospholipid composition of the endosymbiont and the mitochondrion, an organelle with symbiotic origin, which is used as a comparative model in our study.

Supported by: CNPq, FAPERJ and FUJB

BQ24 - PROTEIN DEPHOSPHORYLATION MAY BE INVOLVED IN *TRYPANOSOMA CRUZI* DIFFERENTIATION

Tonelli, R.R., Silber, A.M., Colli, W. and Alves M.J.M.

Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, SP, Brazil.

Differentiation of the infective trypomastigote form of *Trypanosoma cruzi* to the replicative amastigotes normally occurs in the cytoplasm of infected cells. A preliminary study on the involvement of protein phosphatases type 1 and 2A in the extracellular differentiation of trypomastigotes to amastigotes was undertaken at 37°C and 33°C using the CL-14 clone of *T. cruzi*. Calyculin A, an inhibitor of protein phosphatases 1 and 2A, triggers the transformation of trypomastigotes to amastigotes at neutral pH through epimastigote-like intermediate forms. Treatment of trypomastigotes for 6 hours with 1 nM or 5 nM Calyculin A resulted in the differentiation of more than 50% of trypomastigotes to epimastigote-like forms. After 8 hours, all trypomastigotes were differentiated to amastigotes or epimastigotes-like forms. Taken into account previous results of our laboratory (Almeida-de-Faria et al., Exp. Parasitol. 92, 263-274, 1999) these results suggest that the epimastigote-like form is an intermediate stage in both directions of the amastigote – trypomastigote inter-transformation. Interestingly, metacyclic trypomastigotes from the CL-14 clone submitted to the same treatment with Calyculin A showed no morphological changes.

Financial support: FAPESP

BQ25 - *TRYPANOSOMA CRUZI* TRANSFORMATION INDUCED BY PI-PLC LEADS TO CALCIUM MOBILIZATION AND AMASTIN EXPRESSION

Silva-Jardim, I.[1], Ferraz, D. B.[1], Silva, R. A.[2], Teixeira, S. M. R.[2], Tedesco, A. C.[3] & Ramalho-Pinto, F. J[1].

[1]Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto and [3]Departamento de Química, Faculdade de Filosofia Ciências e Letras, Universidade de São Paulo, Ribeirão Preto, SP, CEP 14.049-900, [2] Departamento de Bioquímica e Imunologia, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, MG. e-mail: ijardim@rbi.fmrp.usp.br

Trypanosoma cruzi trypomastigotes treated with exogenous phosphatidyl inositol-specific phospholipase C (PI-PLC) of *B. thuringiensis in vitro* are rapidly induced to differentiate into round forms very like amastigotes. Upon contact with PI-PLC, morphological changes occur very readily in the parasite. In the present work we have studied the mechanisms underlying these morphogenetic changes, and the signals that are generated and that induce this phenomenon. We have shown that treatment of trypomastigotes of *T. cruzi* with genistein, an inhibitor of protein tyrosine kinase, blocked the differentiation of the parasites. The inhibitor of protein kinase C, calphostin C and the inhibitor of adenylate cyclase, 2,5-dideoxyadenosine, do not block the transformation. The Ca^{2+} response induced in trypomastigotes upon contact with PI-PLC is discrete, with a slight variation in the intracellular calcium concentration of the parasite. Pre-treatment of trypomastigotes with calcium chelator BAPTA/AM also have a slight effect in the transformation of trypomastigotes into amastigotes. We also show, by Northern-blotting, that the treatment of trypomastigotes of *T.cruzi* with PI-PLC induces the expression of amastigote-specific genes. After six hours of the treatment with PI-PLC, the amastigote-like forms express the mRNA for amastin, a *T. cruzi* amastigote-specific gene. Taken together, these data indicate that *T. cruzi* transformation induced by PI-PLC activates protein tyrosine kinases pathway with calcium mobilization and expression of amastigote-specific genes.

Financial Support: CAPES

BQ26 - HEME REQUIREMENT AND ITS POSSIBLE INTRACELLULAR TRAFFIC IN *TRYPANOSOMA CRUZI* EPIMASTIGOTES.

Lara, F.A.[¶]; Laranja, G.A.T.*; Figuêiredo-Oliveira, F.[+]; Sant'Anna, C.[#]; Cunha-e-Silva, N.[#]; Oliveira, P.L.[¶], Coelho, M.G* and Paes, M.C.*

[¶] Departamento de Bioquímica Médica, Instituto de Ciências Biomédicas – UFRJ; *Departamento de Bioquímica, Instituto de Biologia – UERJ; [#] Lab. de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica – UFRJ; [+] Centro Federal de Educação Tecnológica RJ - Química

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

Supported by FAPERJ, CNPq, PADCT, and HHMI (to PLO) and PRONEX.

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BQ27 - L-GLUTAMINE INDUCES *TRYPANOSOMA RANGELI* DIFFERENTIATION *IN VITRO*

Koerich L.B., Romanha, A.J., Grisard E.C., Steindel M.

Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Catarina, Caixa postal 476, Florianópolis, SC, Brazil, 88040-900. Florianópolis - SC. e-mail:ccb1mst@ccb.ufsc.br

The difficulty in obtaining infective trypanosomes has been a break point for the study of several aspects of *Trypanosoma rangeli* infection in mammals. For that, our group has developed a simple method to induce *T. rangeli* differentiation *in vitro,* obtaining differentiation rates over 75%. However, the mechanism or mechanisms and factors involved in the differentiation phenomenon are barely known. In this study, we have identified the amino acids responsible for induction of *T. rangeli* differentiation *in vitro*. For that, epimastigotes of *T. rangeli* Choachi strain harvested in LIT medium were washed twice in PBS and cultivated in the MEM amino kitÒ (Gibco, USA), which is basically a DMEM medium, depleted of all amino acids. The assays were performed three times in triplicate in 1.5 ml micro tubes using 10^6 parasites/mL at 26°C by adding one single amino acid at a time. Differentiation was assessed in each testing medium at 0, 2, 4 and 6 days of incubation by random counting of 300 parasites in Giemsa stained smears to determine the number of trypomastigotes. On the 6^{th} day of incubation, parasites cultivated in the presence of L-glutamine, presented a differentiation rate of 83.8±8.9%, while in the control medium (DMEM plus all amino-acids) the differentiation rate was 75.7±4.5%. Among the other 12 amino acids tested, significant lower differentiation or higher mortality rates of the parasites were observed. These herein described results suggest that L-glutamine plays an important role on *T. rangeli* differentiation *in vitro*, probably involved in the polyamines pathway. The study and comprehension of the *T. rangeli* differentiation process *in vitro* is of great importance for the study of the parasite biology as well as for comparative studies with *T. cruzi.*

Supported by CNPq, UFSC and FIOCRUZ.

BQ28 - THE METABOLIC CONTRIBUTION TO *HERPETOMONAS ROITMANI* WITH A BACTERIUM-LIKE ENDOSYMBIONT IN THE CYTOPLASM.

Binda Jr., A. L.*; Freitas Lemes, A.P.*; Evangelista Fiorini, J.**; Schneedorf, J.M.

Biologia e Fisiologia de Microorganismos, UNIFENAS; Faculdade de Ciências Médicas, UNIFENAS; Medicina, UNIFENAS.

The Tripanosomatidae family has singular importance due to its capacity to infect many organisms even men's, which results in efforts for its eradication, that is difficult to achieve due to lack of knowledge. For biological studies of the family, the group Hepertomonas has been regarded as a good comparative model. The incoming work evaluates the ergogenic potential in *Herpetomonas roitmani* through conduction microcalorimetry. The protozoons were cultivated in Roitmani, to keep them for microcalorimetry and for its control. To find the cure, several changes of blood cultures, containing clorafenical, to Roitman's chemically defined minimum were done. We also did experiments in microcalorimetry using 0,5 ml of energetic substratum, 0,5 ml of physiological serum and 0,8 ml of cellular suspension. The statistic analysis was conducted based on linear minimum squares. The heat conduction resulted in a value of 29,3 -/+ 12,9 pw/cell of heat liberation by the protozoon Trypanosomatid with a bacterium-like endosymbiont in the cytoplasm (R=0,794). The experiments show that the medium value of the produced heat by *Herpetomonas roitmani* matches with other reported values from other cells such as the Hybridoma 30-50 pW/cell;3T3, 53 pW/cell, and is superior than the human ordinary cells.

BQ29 - CLONING, EXPRESSION AND CHARACTERIZATION OF THE CYSTEINE PROTEASES FROM *TRYPANOSOMA BRUCEI BRUCEI* (BRUCIPAIN) AND *LEISHMANIA (L.) DONOVANI*

Tatiana F. R. Costa, Flavia C. G. dos Reis, Julio Scharfstein and Ana Paula C. de A. Lima.

Instituto de Biofísica Carlos Chagas Filho, CCS, Universidade Federal do Rio de Janeiro, RJ, Brasil.

Pathogenic trypanosomatids express high levels of papain-like cysteine proteases (CPs), which are important for their pathogenicity and survival in the host. The use of potent synthetic irreversible CP inhibitors in experimental Chagas' Disease and leishmaniasis has helped the validation of these enzymes as promissing targets. The development of more effective and selective inhibitors depends on the detailed structural and functional characterization of these molecules. The only trypanosomatid CP characterized at the structural level is the one of *Trypanosoma cruzi*, cruzipain, obtained in its recombinant form truncated at the C-terminus. In trypanosomatids, these proteases are usually encoded by polymorphic gene families, whose members may present variable kinetic properties. Therefore, the fine characterization of a particular member requires its functional expression in a heterologous system, which has been recently achieved for the CPs of *Leishmania (L.) mexicana* and of *Trypanosoma brucei rodhesiense* (rodhesain). Here, we report the expression of functional CPs from *Trypanosoma brucei brucei* (brucipain) and from *Leihmania (L.) donovani*, followed by a preliminary characterization of their biochemical properties. Degenerated oligonucleotides based on the sequence of rodhesain were used as primers in PCR reactions with genomic DNA of *T. brucei* as a template, leading to the amplification of a single fragment, which presents 95% similarity with rodhesain. A gene fragment spanning the pro and central domains of brucipain were cloned in the vector pQE30 for the *E. coli* expression of a fusion protein bearing a 6 histidine residue-tag at the N-terminus of the enzyme. Recombinant pro-brucipain, obtained as inclusion bodies, was denatured in urea buffer, purified in Ni-agarose resin and refolded "in vitro". Active enzyme was produced upon incubation of the precursor forms at low pH and low temperature and subsequently affinity purified in the tio-propyl sepharose resin. Using a similar strategy, complementary oligonucleotides based on of the CP from *L. (L.) donovani* (GenBank), were used in PCR reactions and the recombinant mature enzyme was obtained as described above. The preliminary characterization of brucipain and of r- CP revealed that they efficiently hydrolize Z-Phe-Arg-MCA. Interestingly, brucipain displayed strong substrate inhibition when assayed with this substrate at room temperature, a feature described with cruzipain. Similarly to cruzipain, brucipain did not display substrate inhibition when assayed at 37ºC, suggesting that it also bears a second substrate binding site at the central domain, which is modulated by temperature. If present with natural substrates, the parasite could use temperature-dependent substrate inhibiton to modulate enzyme activity when it changes from the invertebrate to the vertebrate host. Detailed characterization of the enzymes' substrate specificity using long synthetic substrates is now underway.

Supported by CNPq/Universal and FAPERJ.

BQ30 - POTENT INHIBITION OF TRYPANOSOMATID CYSTEINE PROTEASES BY THE PRO DOMAIN OF CRUZIPAIN

dos Reis, F.C.G., Costa, T.F.R., Scharsftein, J. and Lima, A.P.C.A.

Instituto de Biofísica Carlos Chagas Filho, CCS, Universidade Federal do Rio de Janeiro, RJ, Brasil.

Pathogenic protozoa bear high levels of papain-like cysteine proteases (CPs),

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which have been associated with parasite virulence and survival. Similarly to their mammalian counterparts, these enzymes are expressed as inactive precursors, which undergo maturation by proteoliytic removal of the pro segment. Although there is a large body of information on the biochemical properties of trypanosomatid CPs, little is known about the mechanisms controlling their activity "in vivo". The rate of zymogen processing could be one way of modulating the levels of mature enzyme. In mammalian cells, it was demonstrated that free intact pro segment of lysosomal CPs, cathepsins L and B, selectively inhibit the respective mature enzyme with high affinity in a pH-dependent manner. It has been suggested that, if intact pro segment is liberated upon pro-enzyme processing in vivo, it could act as an inhibitor of mature enzyme until the complex is delivered to acidic compartments. A similar mechanism could regulate the levels of active CPs in trypanosomatids. Along these lines, it has been recently demonstrated that the pro segment of congopain, the CP from *Trypanosoma congolense*, inhibits congopain and cruzipain (the CP from *Trypanosoma cruzi*) with moderate affinity. The mapping of the inhibitory regions within the pro segment of congopain using synthetic overlapping peptides associated the YHNGA sequence (partially conserved among trypanosomatid CPs) with the inhibitory activity. In contrast with mammalian CPs, where pro domain-dependent inhibition is highly selective, it is possible that there is pro segment cross-inhibition among CPs of different trypanosomatids Here, we set out to investigate the inhibitory properties of the pro segment of cruzipain. A DNA fragment corresponding to the pro segment of cruzipain (Cys X-Gly X) was generated by PCR using complementary oligonucleotides and subsequently cloned into the pQE30 expression vector. The recombinant pro segment containing a 6 histidine resisue–tag at its N-terminus was produced in *E. coli*, purified in affinity resins and tested for inhibitory activity towards cruzipain, using Z-Phe-Arg-MCA as a substrate. We demonstrate that the recombinant pro domain of cruzipain strongly inactivates mature enzyme (K_i = 0.03 nM) at pH 6.5. This result indicates that if the pro segment is present intact in reservosomes, or liberated to the extracellular environment, it could act as a key regulator of cruzipain function. Interestingly, we observed that the recombinant protein also potently inactivates recombinant brucipain (the CP of *T. brucei*), the CP of *Leishmania donovani*, as well as lysates of other trypanosomatids such as *T. rangeli* and *Chritidia fasciculata*, while it did not inhibit papain, and cathepsins L or B. The properties of this molecule could be exploited as an alternative to generate a potent selective inhibitor of trypanosomatid CPs.

Supported by CNPq, FAPERJ.

BQ31 - SPECIFICITY OF SERINE PROTEASES OF *L. (L.) AMAZONENSIS*

Lopez, R.E.S. & De Simone, S.G.

Laboratório de Bioquímica de proteínas e Peptídeos- Departamento de Bioquímica e Biologia Molecular – IOC-FIOCRUZ- Rio de Janeiro-RJ

AIMS: Serine protease of parasites play crucial roles in their physiology and in the interaction with their hosts. The inhibition of these enzymes by specific protease inhibitors can interfere in the process of invasion and survival of the parasites within the hosts. Serine proteases of *Leishmania amazonensis* were characterized in our laboratory and their specificity was analysed with the purpose of developing specific inhibitors which could interfere in the parasite physiology.

MATERIALS AND METHODS: Serine proteases were purified from cellular extracts and cell-free extracts of *L. amazonensis* promastigotes using affinity chromatography on aprotinin-agarose and high performance liquid gel filtration chromatography. The enzymatic specificity was analysed using synthetic peptides. The enzyme hydrolyzed substrates in basic pH alkaline range at 28°C.

Results and Conclusions: The enzymes have shown greater specificity for peptides containing aromatic and aliphatic groups at P1 position, such as leucine, valine, alanine and tyrosine. The results showed that serine proteases from *Leishmania (L.) amazonensis* have a similar enzymatic specificity chymotrypsin.

Financial support: FAPERJ, FIOCRUZ, CNPq

BQ32 - DIFFERENTIAL PROTEINASE PROFILES OF *TRICHOMONAS VAGINALIS* ON A WELL-ESTABLISHED STRAIN AND A FRESH ISOLATE

Souza-Silva F[1], Alves CR[1], Silva-Filho FC[2], Melo-Braga MB[2], Rocha-Azevedo B[2] and De Jesus JB[1]

[1]Instituto Oswaldo Cruz, Departamento de Bioquímica e Biologia Molecular FIOCRUZ, Av. Brasil, 4365 Manguinhos, CP 926, 21045-900, Rio de Janeiro, Brasil. [2]Instituto de Biofísica Carlos Chagas Filho, UFRJ, Rio de Janeiro, RJ, Brasil

The parasitic protozoan *Trichomonas vaginalis* is the causative agent of human trichomoniasis, a common infection of the urogenital tract, and exerts its pathogenic effect when interacting with the surface of epithelial cells. This infection is globally considered one of the most frequent sexually transmitted diseases, with approximately 180 to 200 million cases annually (Rendón-Maldonado *et al*., 1998, *Exp.Parasitol*., 89: 241-250). Several molecules have been identified as virulence factors involved in the mechanism of tissue damage by *T. vaginalis* i. e. adhesins, a cell-detaching factor, a laminin-biding receptor, pore-forming proteins and proteinases. *T. vaginalis* proteinases have been related to nutrient acquisition, immune evasion and cytotoxicity. In this work we aim to characterize the protease expression profile among microorganisms of a fresh isolate of *T. vaginalis* (FMV1 strain) and of a long-term culture (FF28JT-Rio strain). Proteinase activity was detected on immobilized gelatin substrate copolymerized with polyacrilamide gel electrophoresis and in solution with a chromogenic substrate. Both FF28JT-Rio and FMV1 strains were cultivated in TYM medium supplemented with 10% fetal bovine serum and maintained at 37 °C for 24 hours. Parasites were washed in PBS pH 7.2 and lysed in 10 mM Tris-HCl pH 6.8 containing 1% of Triton X-100 (30 minutes, 4 C). The insoluble fraction was removed by centrifugation (12 000 g, 30 minutes, 4 C) and the enzymatic activity was evaluated in the soluble fraction. Qualitative electrophoretical analyses have showed that a constant pattern of proteinase bands with molecular mass between 93 – 8.7 kDa might be detected in both parasite samples in acid pH. However, a differential profiles was detected in both parasite extract: 93.7, 81.8, 62.4, 52.0, 41.6, 31.1, 22.3, 11.8 and 8.2. kDa from FF28JT-Rio and 89.1, 71.3, 65.3, 40.0, 26.6, 19.2, 13.1 and 8.7 kDa from FMVI parasite. In the inhibition assay we observed that only the cysteine-proteinase inhibitor (E-64) had an effect on total proteinase band profile of both parasites. An additional qualitative assay was performed with synthetic peptide substrate. The specific chromogenic substrate pGlu-Phe-Leu p-nitroanilide; (0,1mM) was digested in 50mM Sodium Acetate pH5.0 containing 1mM dithiotreitol, 10mg of soluble protein fraction in final volume of the 500ml (25 C, 10 minutes). In this last experiments it was also possible to quantify the activity of protienase on the FF28JT-Rio strain (36,0 nmol minute^{-1} mg of protein^{-1}) and on FMV1strains (35,0 nmol minute^{-1} mg of protein^{-1}). The implications of preliminary results are discussed.

Financial Support: CNPq, FAPERJ, FINEP and PRONEX.

BQ33 - IDENTIFYING SERINE AND CYSTEINE PROTEINASES IN *ACANTHAMOEBA* ISOLATES

Ferreira, G. A. & Alfieri, S. C.

Departamento de Parasitologia, ICB-II, USP. Av. Prof. Lineu Prestes, 1374, CEP 05508-900, São Paulo, SP, Brasil.

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Free-living amebae of the genus *Acanthamoeba* multiply as phagotrophic trophozoites and encyst under unfavorable conditions. *Acanthamoeba* spp. have been identified as the causative agents of granulomatous amebic encephalitis and amebic keratitis in man (Marciano-Cabral & Cabral, Clin. Microbiol. Revs.16: 273, 2003). Tissue damage and invasion by the amebae still remain poorly understood, but contact-dependent cytopathic effects and proteinase secretion seem to be involved. We have shown that *A. polyphaga* trophozoites (ATCC 30461) constitutively secrete multiple cysteine proteinases (CPs) and serine proteinases (SPs) (Alfieri *et al.*, J. Parasitol. 86: 220, 2000). We have extended the observations to several *Acanthamoeba* isolates (*A. castellanii* ATCC 30234, 30011, 30868; *A. polyphaga* ATCC 30871, 30872, 30873, 30461), including three from brazilian cases of keratitis (U/E 2, U/E 8x, U/E 10). Proteinase activities were monitored by azocasein assays and gelatin-containing SDS-polyacrylamide gels, using as enzyme source cell lysates and medium conditioned for 24 hours by trophozoites (*Acanthamoeba* conditioned medium, ACM).

Azocasein hydrolysis was detected over a broad pH range (4.0-10.0) in ACM, and with maximal rates at pH 6.0 and above; substrate hydrolysis at near neutral/alkaline pH was potently inhibited by PMSF, thus indicating the involvement of SPs. Serine proteinases were detected in low levels in cell lysates; assays performed at pH 5.0 with 2 mM DTT indicated high CP activity in the latter.

SPs were identified in ACM following electrophoresis in gelatin gels and incubation in 0.1M Tris-HCl, pH 8.0. In *A. castellanii*, there were bands common to all isolates (50, 60 and 100 kDa) and others (55 and 170 kDa) visualized only in isolates 30011 and 30234. In *A. polyphaga*, in addition to a band near 100 kDa common to isolates 30871, 30873, and 30461, SPs distributed as follows: 50, 57, 60 kDa (30871); 44, 80 kDa (30872); 47, 51, 56 kDa (30873); 52, 60 kDa (30461). In the three clinical isolates, SPs resolved identically to the enzymes of *A. polyphaga* 30461. SPs apparently equivalent to those identified in ACM were detected in cell lysates; visualization of tracks of activity in gels fixed and stained immediately after running indicated enzyme activation during electrophoresis.

Blockage of SPs with PMSF prior to electrophoresis and gel incubation at pH 3.8 with 2 mM DTT was the condition used to examine CPs in activity gels (Alfieri et al., 2000). Bands inhibited by E-64 and thus associated with CPs, although more intensely visualized in cell lysates, were also identified in ACM: four were detected in *A. castellanii* 30011 and 30234 (42, 45, 75, 116 kDa), and five (36, 45, 66, 75, 116 kDa) in isolate 30868. There were differences between *A. polyphaga* isolates, but all displayed activities around 70 and 100 kDa. Again, in the clinical isolates, CPs resolved identically to the enzymes of *A. polyphaga* 30461.

Support: CNPq

BQ34 - CLONING AND EXPRESSION OF A RECOMBINANT CYSTEINE PROTEASE (TFCP1) FROM *TRITRICHOMONAS FOETUS*

Melo-Braga, M. B.[1]; Lima, A. P. C. A.[2]; Silva, R[3]; Penha, L. P.[2]; Rocha-Azevedo, B.[1]; Rondinelli, E.[3]; Scharfstein, J[2]; Silva-Filho, F. C.[1]

[1]Laboratório de Biologia da Superfície Celular, [2]Laboratório de Imunologia Molecular & [3]Laboratório de Metabolismo Macromolecular Firmino Torres de Castro, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro – RJ, 21949-900, Brasil. e-mail: mariane@biof.ufrj.br

Tritrichomonas foetus, the causative agent of bovine trichomonosis, inhabits the mucosal surfaces of urogenital cavities where it is found adhering to the subjacent epithelium. This parasitic cytoadhesion may result in cytotoxicity to the host tissue. Previously data obtained in our laboratory indicate that cysteine proteases (CP) might play an important role in the disruption of epithelial monolayers by *T. foetus*. In order to further study the biochemical and biological attributes of CPs in *T. foetus* we have designed experiments having as tool a recombinant cysteine protease in *Escherichia coli*.

Using primers based upon the sequence of the gene *tfcp1* (accession number U13153) we obtained a fragment of 928 kb by PCR amplification of genomic DNA of parasites from the K strain of *T. foetus*. The sequence corresponds to an open reading frame which encodes a protein of 35 kDa showing 56% similarity with human cathepsin L. Comparison with the original sequence led to the identification of three aminoacid divergences that may correspond to strain variation. This fragment was cloned into pQE30 vector and transfected into *E. coli* M15. A recombinant 6xHis tagged protein of approximately 40 kDa was obtained after induction of expression by adding IPTG in bacteria culture medium and purification from *E. coli* extracts using Ni-affinity chromatography. Experiments are now being carried out to determine ideal refolding conditions for the recombinant enzyme and to obtain policlonal antisera against TFCP1.

Supported by: CAPES-MEC, CNPq, FAPERJ, FUJB-UFRJ & MCT-PRONEX

BQ35 - *LEISHMANIA (L.) MAJOR* INHIBITOR SCREENING GUIDED BY ADENINE PHOSPHORIBOSYLTRANSFERASE (APRT) INHIBITIONS

Silva M [1], Guedez B. D.[1], Napolitano, H. B. [1], Rocha W.C.[2], Almeida A.L.C[2], Vieira P.C.[2,] Oliva G.[1] and Thiemann O.H. [1]

[1] Protein Crystallography and Structural Biology Group, IFF/IFSC/USP, São Carlos, 13560–970, SP, Brasil. [2] Chemistry Department, UFSCar, São Carlos, Brasil. marcios@if.sc.usp.br

Leishmaniasis is a serious disease caused by parasites of the order Kinetoplastida. According to the World Heath Organization (WHO, 1998), 88 countries are affected, with 12 million people infected, and approximately 350 million at risk of infection. The need for new drugs for the treatment of leishmaniasis comes from a lack of safe drugs and the serious secondary effects observed in the available chemotherapy. In order to look for new anti-*Leishmania* drugs we used recombinant APRT from *L. tarentolae*, as model system, to screening the inhibitory capacity of both plant and marine animal extracts through exploration the Brazilian biodiversity. This investigation has been done using an easy and fast spectrophotometric enzymatic assay. The best inhibitors of APRT were selected to be used in an *in-vivo L. (L.) major* inhibition study. The obtained results from enzyme screening lead us to identification of eighteen promising compounds. The best three IC_{50} values from selected molecules are 151 μM, 142 μM and 50 μM and the respective *L. (L.) major* killing capacity LD_{50} are 109 μM, 96,5 μM and 30 μM. The enzyme APRT and the three best inhibitors had their structures determined by X-ray crystallography. In a next step we intend to use these selected inhibitors in experiments of rational drug design based on structural information. The approach includes: co-crystallization of protein-inhibitor, computational molecular docking and QSAR (Quantitative Structure Activity Relationships). The information will be used to orient the molecular synthesis of new compounds toward the improvement of the inhibitory activity.

Supported by: FAPESP, CEPID, PRONEX, HHMI and WHO/TDR

BQ36 - INHIBITION OF *TRYPANOSOMA CRUZI* GROWTH BY *PTERODON PUBESCENS* OILY EXTRACT

Braga, S G., Carvalho, F.C.T, Silva, M.C.C., Coelho, M.G.P, Todeschinini, A. R.

Departamento de Bioquímica, Instituto de Biologia, Universidade do Estado do Rio de Janeiro, Boulevard 28 de Setembro 87, 20551-013, Rio de Janeiro,RJ, Brasil

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Trypanosoma cruzi is the etiologic agent of Chagas´ disease, an endemic disease in Central and South America. Chemotherapy of this disease is still very unsatisfactory, being based in nitrofurans and nitroimidazoles. These compounds are inadequate due to frequent toxic side effects and limited efficacy having little or no activity in the chronic phase of the disease. These facts show us the urgency for the development of new drugs more effective and less toxic for Chagas´ disease. *Pterodon pubescens* Benth (leguminosae), known as Sucupira branca is a native tree specie of Brazil and its seeds are used as hydroalcoholic infusion presenting anti-rheumatic, analgesic, anti-inflammatory and cercaricide properties. Toxicological studies demonstrated that the *P. pubescens* seeds extracts did not present acute or sub-acute toxicity. Geranylgeraniol and related substances (14,15-epoxygeranylgeraniol and 14,15-dihydro-14,15-dihydroxygeranylgeraniol) have been associated to the cercaricide activity of Pterodon pubescens oil. Isoprenoids are involved in cell proliferation and differentiation, and much work is being done nowadays to study these compounds, especially farnesol and geranylgeraniol, that are also involved in post-translational prenylation of proteins, facilitating protein-protein interactions and membrane-associated protein trafficking. In this work we study the effects of oleaginous extract of *P. pubescens* seeds (Ppoe), the hexanic fraction (Hex) and geranilgeraniol in the growth of *T. cruzi* epimastigotes from Y strain. Ppoe was obtained by maceration of *Pterodon pubescens* seeds in ethanol at room temperature for 15 days. A hexanic extract (Hex) from Ppoe was obtained by liquid-liquid extraction. Geranilgeraniol was further obtained from Hex by HPLC in a C8 column and characterized by GC-MS and NMR. The epimastigotes were grown in BHI, with 10% SFB, 10mg l^{-1} hemin and 20mg l^{-1} folic acid for 25ºC for 7 days in the presence and in the absence of Poep or H2. The EOPp, Hex and H2 presented a dose-dependent inhibition of epimastigotes growth with an IC50 of 12.31; 13.64 and 31.84 mg/ml respectively. Studies to the mechanism of action of the geranilgeraniol in the cellular division of the *T. cruzi* are in course in our laboratory.

Acknowledgments: CNPQ, FAPERJ.

BQ37 - LEISHMANICIDAL ACTIVITY OF POLYPHENOLICS-RICH EXTRACT FROM HUSK FIBER OF *COCOS NUCIFERA* LINN. (PALMAE)

Mendonça-Filho RR [a,b], Rodrigues IA [a], Alviano DS [a], Santos ALS [a], Soares RMA [a], Alviano CS [a], Lopes AHCS [a] & Rosa MSS [a]

[a] Instituto de Microbiologia Prof. Paulo de Góes (IMPPG), Centro de Ciências da Saúde (CCS), Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, 21941-590, Brazil, [b] Instituto de Química, Programa de Pós-graduação em Ciência de Alimentos, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, 219491-590, Brazil.

The available therapy for leishmaniasis, which affects 2 million people per annum, still causes serious side effects. The polyphenolics-rich extract from the husk fiber of *Cocos nucifera* Linn. (Palmae) presents antibacterial and antiviral activities, also inhibiting the lymphocyte proliferation, as shown by our group in previous works. In the present study, the *in vitro* leishmanicidal effects of *C. nucifera* on *Leishmania (L.) amazonensis* were evaluated. The minimal inhibitory concentration of the polyphenolics-rich extract from *C. nucifera* to completely abrogate the parasite growth was 10 mg/ml. Pretreatment of peritoneal mouse macrophages with 10 mg/ml of C. *nucifera* polyphenolics-rich extract reduced in approximately 44% the association index between these macrophages and *L. (L.) amazonensis* promastigotes, with a concomitant increase of 182% on nitric oxide production by the infected macrophage in comparison to non-treated macrophages. These results provide new perspectives on drug development against leishmaniasis, since the extract of *C. nucifera* at 10 mg/ml is a strikingly potent leishmanicidal substance, which inhibited the growth of both promastigote and amastigote developmental stages of *L. (L.) amazonensis* after 60 min, presenting no in vivo allergenic reactions or in vitro cytotoxic effect in mammalian systems.

Financial Support: CNPq, FAPERJ, FUJB, CAPES.

BQ38 - α-TOMATINE TOXICITY ANALISIS ON TRYPANOSOMATIDS PARASITES OF PLANTS AND INSECTS.

Evangelista A.F.[1], Kavati E.A.[1], Menolli R.A.[1*], Graça V. K.[1], Itow Jankevicius S.[1], Jankevicius J.V. [2]

[1]Universidade Estadual de Londrina – UEL, Londrina, Paraná, Brasil. [2]Universidade Norte do Paraná - UNOPAR, Londrina, Paraná, Brasil. *E-mail: ramenolli@hotmail.com

The plants accumulate a great variety of secondary metabolites that have important functions on plant protection against pathogens. The glycoalkaloids are a class of substances that are involved in this protection and a better understanding about its role against diverse plant parasites has great practical interest. The glycoalkaloid α-tomatine exists in high concentrations in green tomato fruits (*Lycopersicum esculentum*), whereas in the ripe fruits it is changed to its aglycone form, tomatidine. In the case of *Phytomonas serpens*, a trypanosomatid parasite of tomato fruits, preliminary results showed that its development occur only in ripe fruits, what could be an evidence of involvement of α-tomatine in the defense of the tomato plant. Its protective function against fungal phytopathogens is well known and here it was evaluated against trypanosomatid parasites of plants and insects. We used logaritmic fase culture forms in GIPMY medium of seven strains of lower Trypanosomatids (genera *Phytomonas*, *Herpetomonas*, *Crithidia* and *Leptomonas).* To determine the 50% lethal dosis (LD50), we mixed equal volumes of 10^7 of the various culture forms with concentrations between 10^{-3} and 10^{-8} M of α-tomatine in PBS (pH 7,2, 150 mM with 10% ethanol), during thirty minutes at room temperature. The test was evaluated by the% of motility of the protozoan culture forms observed with optical microscopy in a Newbauer chamber. The results after the LD50 determination showed differences between the strains, with the LD50 oscilating between 10^{-3} and 10^{-5}M of á-tomatine. The same tests realized with the tomatine aglycone form, tomatidine, showed little toxicity on the same evaluated trypanosomatid culture forms. The mode of action on membranes and the antifungic action of α-tomatine is well known, but with protozoans, is poorly understood and this is the first step to clarify its role in plant defense against trypanosomatid phytopathogens.

Financial support: CNPq and CAPES

BQ39 - A COMPARATIVE STUDY OF MESOIONIC DERIVATIVES AGAINST *LEISHMANIA (L.) CHAGASI* AND *LEISHMANIA (V.) BRAZILIENSIS*

Fajardo-Bonin R[1]., Curvelo G. S[1]., Echevarria, A[2]., Leon, L. L[1].and Canto-Cavalheiro M.[1].

[1] Departamento de Imunologia, Instituto Oswaldo Cruz, Fiocruz; [2] Departamento de Química, Universidade Federal Rural do Rio de Janeiro, UFRRJ

In a previous study searching for new and highly effective antileishmanial drugs with low toxicity, we reported the *in vitro* activity of a series of mesoionic derivatives against *L. (L.) amazonensis* (Silva et al., 2002). As a part of our research program on chemotherapy against diseases caused by *Leishmania* spp., we decided to assay some 1,3,4-thiadiazolium-2-aminide class of mesoionic derivatives against other two different species of *Leishmania*, which has been associated to the others clinical forms of disease. The species used in this work were *L. (V.) braziliensis*

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that usually caused mucocutaneous disease and is endemic in the state of Rio de Janeiro and *L. (L.) chagasi* that is the causal agent of visceral leishmaniasis. These compounds, 4-phenyl-5-(4' and 3'-methoxy-cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chlorides were assayed against *L. braziliensis* (MCAN/BR/98/R619 strain) and *L. (L.) chagasi* (MCAN/BR/97/P142 strain) promastigotes and using Pentamidine Isethionate as reference drug. Parasites in the late log phase culture were incubated with several concentrations of the drugs solubilized in dimethyl sulphoxide (DMSO) and then counted in a Neubauer's chamber. Controls without the drugs and with DMSO were done in parallel. The preliminary results showed that mesoionic compounds such as 4'-methoxy and 3'-methoxy were effective against both *L. (V.) braziliensis* and *L. (L.) chagasi*. However, these compounds were about seven fold more effective against *L. (L.) chagasi* than *L. (V.) braziliensis* in promastigotes forms. Concerning the *L. (L.) amazonensis* promastigotes (MHOM/BR/77/LTB0016 strain), data from our previous work showed significantly difference in the sensitivity between both 3'- and 4'-methoxy mesoionic compounds; the 3'-methoxy derivative (IC_{50}/24=0.02±0,01µg/mL) was more efficient than 4'-methoxy compounds (IC_{50} /24=0.07±0.01 µg/mL). The present data showed that comparing the effect of those derivative against the three species and it was observed that the most sensitive was *L. (L.) amazonensis,* while *L.* chagasi (4'-OCH3 IC_{50}/24= 1,25±0,35 µg/mL; 3'-OCH3 IC_{50} /24= 1,73±0,27µg/mL) had a sensitivity between *L. (L.) amazonensis* and *L. (V.) braziliensis*, the last having the highest (4'-OCH3 IC_{50} / 24 = 6,5 ±0.70 µg/mL; 3'-OCH3 IC_{50} /24 = 8,3±1.55 µg/mL) Furthermore, in a comparative analysis using four mesoionic salts against *L. (L.) amazonensis* and *L. (L.) chagasi* promastigotes, it was observed that again *L. (L.) amazonensis* had lower IC_{50}/24 (4'-H IC_{50}/24 =0,18±0,01 µg/mL; 4'-OEt IC_{50}/24 =0,65±0,09 µg/mL; 4'-F IC_{50}/24 =0,38±0,03 µg/mL; 3'-Cl IC_{50}/24 =0,21±0,01 µg/mL) than *L. (L.) chagasi* (4'-H IC_{50}/24 =3,19±0,65 µg/mL; 4'-OEt IC_{50}/24 =4,75±0,51 µg/mL; 4'-F IC_{50}/24 =2,26±0,09 µg/mL; 3'-Cl IC_{50}/24 =2,261±0,09 µg/mL). The difference of effectiveness would be explained by the differences in the culture conditions such as higher concentration of fetal calf serum, since some amount of drug could be linked to the serum albumin, resulting in a decrease in the drug availability. Besides, it would be expected that the metabolism of the three species should be different.

Supported by CNPq/PDTIS/PAPES/IOC-FIOCRUZ

BQ40 - ANTILEISHMANIAL ACTIVITY OF NEROLIDOL

Denise C. Arruda, Alejandro M. Katzin and Silvia R. B. Uliana

Departamento de Parasitologia – Instituto de Ciências Biomédicas – Universidade de São Paulo, Avenida Professor Lineu Prestes, 1374, ICB2, Cidade Universitária, SP, SP, Brasil, CEP:05508-900. srbulian@icb.usp.br

Isoprenoids are essential compounds required for cell proliferation and differentiation. Isoprenoid biosynthesis in most eukaryotes derives from the mevalonate pathway. From acetyl-coenzyme A as the main substrate, most organisms synthesize hydroxymethylglutaryl-CoA (HMG-CoA) which is reduced to mevalonate by HMG-CoA reductase. Mevalonate is converted into isopentenyl pyrophosphate (IPP) which generates geranyl pyrophosphate and farnesyl pyrophosphate, the main precursors of polyisoprenoids such as dolichol, ubiquinone, cholesterol and prenyl groups transferred to prenylated proteins. The inhibition of isoprenoid biosynthesis leads to suppression of cell growth and death.

Terpenes are isoprenoids found in oils derived from fruits and herbs and their antibactericidal, antitumoral and antiprotozoal activity have been under study. The effect of nerolidol against *Plasmodium falciparum* has been shown to be due to the interference in the isoprenoid biosynthetic pathway (Macedo et al., FEMS Microbiol. Let., 207:13-20, 2002).

To investigate a possible leishmanicidal activity of nerolidol, in vitro cultures of *L. (L.) amazonensis* promastigotes and amastigotes were grown in the presence of increasing concentrations of the drug. The growth of both life cycle stages was arrested by nerolidol with IC50s of 90 mM for promastigotes and 53 mM for amastigotes. The effect of nerolidol was also demonstrated against *L. (L.) major* (IC50 67 mM), *L. (V.) braziliensis* (IC50 70 mM) and *L. (L.) chagasi* (IC50 73 mM) promastigotes and *T. cruzi* epimastigotes (IC50 105 mM).Metabolic labeling of promastigotes and amastigotes of *L. (L.) amazonensis* with [2-^{14}C] mevalonate followed by analysis of the hexane fraction by HPLC, showed that the synthesis of dolichol and of the isoprenic chain of ubiquinone are 91% inhibited after treatment with nerolidol 30 mM. These results suggest that terpenes may have great potential in the development of new antileishmanial chemotherapeutic agents.

Supported by: FAPESP

BQ41 - PARTIAL CHARACTERIZATION OF A UDP-GLCNAC: POLYPEPTIDE A-*N*-ACETYLGLUCOSAMINYLTRANSFERASE IN *CRITHIDIA FASCICULATA*.

Santiago, A.P.S.A.; Previato, J.O.; Heise, N. and Mendonça-Previato, L.

Laboratório de Glicobiologia, IBCCF, UFRJ.

In *Trypanosoma cruzi*, the first step of mucin-like molecules glycosylation occurs in the Golgi complex and consists in the transfer of *N*-acetylglucosamine (GlcNAc) unit from UDP-GlcNAc to threonine (Thr) residues, in a reaction catalyzed by a polypeptide *O*-a-*N*-acetylglucosaminyltransferase (*Previato et al.*, 1998). In contrast, the O-glycans of mammal's mucins are linked to hydroxylated aminoacids Thr and serine (Ser) in the peptide backbone through a a-*N*-acetylgalactosamine (a-GalNAc) unit. Since polypeptide GlcNAc-transferases have been only characterized in *T. cruzi* and *Dictyostelium discoideum* (*Jung et al.*,1998), we decided to investigate the presence of this transferase activity in *Crithidia fasciculata,* a member of the Trypanosomatidae family. *C. fasciculata* is a monogenetic parasite that normally colonize the gut of various fly hosts, and that can be easily grown axenically. In this study, we detected the polypeptide *O*-GlcNAc transferase activity and optimized it's assay. The enzymatic assay was done using microsomal membranes of *C. fasciculata*, a synthetic peptide $KP_2T_8KP_2$ and UDP-[^{3}H]GlcNAc as acceptor and donor substrates respectively. After purification of the O-glycosylated peptide, the incorporation of [^{3}H]GlcNAc was measured by liquid scintillation counting. The enzyme activity showed to be dependent of incubation time and concentration of enzyme and acceptor substrate. The transference has an optimal pH of 7,5-8,5, an optimal temperature of 20°C and requires Mn^{2+}. The glycosylated $KP_2T_8KP_2$ product is susceptible to base-catalyzed b-elimination, and the efficiency and level of glycosylation were determined with chromatographic methods. These results demonstrated a great efficiency in the transfer reaction of the GlcNAc residue to the synthetic acceptor, with a polypeptide *O*-GlcNAc transferase activity comparable of the one previously described in epimastigotas and trypomastigotes forms of *T. cruzi*.

Supported by: CAPES, CNPq, FAPERJ.

BQ42 - GLYCOSPHINGOLIPIDS (GSLS) FROM AMASTIGOTE FORMS OF *LEISHMANIA (LEISHMANIA) AMAZONENSIS*: CHARACTERIZATION OF A GSLS-BINDING PROTEIN IN MURINE MACROPHAGES

Tanaka AK, Takahashi HK, Straus AH.

Departamento de Bioquímica - Edifício José Leal Prado, Universidade Federal de São Paulo, São Paulo, SP, Brasil

The biologycal cycle of *Leishmania* parasites alternates between a flagellated promastigote in the sandfly midgut and a nonflagellated amastigote stage in the

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mammalian macrophage. Several receptors have been described to *Leishmania* promastigote-macrophage interaction, however the uptake of *Leishmania* amastigote by macrophages remains poorly understood. It has been described two classes of cell-surface receptors that mediate phagocytosis: opsonic and nonopsonic receptors. The *L. (L.) amazonensis* amastigote-macrophage interaction mediated by stage-specific GSLs is a typical example of phagocytosis that do not require parasite opsonization in order to occur macrophage adhesion and internalization, as described by Straus *et al* (J Biol Chem, 1993:268;13723-30). Recently, it was demonstrated in our lab, that a 30 kDa mouse peritoneal macrophage protein interacts either with micelles containing GSLs of amastigote forms of *L. (L.) amazonensis* or with whole amastigote parasites. By Western blotting it was verified that this 30 kDa receptor is not solubilized when macrophages were extracted with PBS containing 0.5% Triton X-100 plus protease inhibitors, remaining in the insoluble fraction. In order to better characterize this receptor, macrophage surface proteins were labeled with biotinamidocaproate N-hydroxysuccinimide ester at 4^0C, and a strong labeling was observed for these proteins confirming their expression at cell surface. Since a variety of lectin-like macrophage receptors have been identified, the purification and the analysis of NH_2-terminal sequence of this 30 kDa macrophage protein are currently been carried out.

Supported by FAPESP and CNPq.

BQ43 - INHIBITION OF TRYPOMASTIGOTES INFECTIVITY BY MONOCLONAL ANTIBODY BST-1, DIRECTED TO EPIMASTIGOTE GIPCS. CROSS-REACTIVITY WITH A HIGH MOLECULAR WEIGHT ANTIGEN PRESENT IN TRYPOMASTIGOTES

Bertini S., Takahashi, H.K. & Straus, A.H.

Department of Biochemistry, Universidade Federal de São Paulo, São Paulo, SP, Brasil

Recently it was a produced in our lab a monoclonal antibody (mAb), termed BST-1, against glycosylinositolphosphoceramides (GIPCs) present in *T. cruzi* epimastigotes. By indirect immunofluorescence a strong parasite labeling was detected with epimastigotes, amastigotes and trypomastigotes. By Western blot it was verified that GIPCs are the only antigens recognized by BST-1 in epimastigotes, on the other hand, when amastigotes and trypomastigotes isolated from culture of VERO cells were analyzed it was observed that besides GIPCs a high molecular weight component (160-200 kDa) was also recognized by the BST-1 antibody. This component was also detected in culture medium of trypomastigotes infected VERO cells.

In order to characterize the synthesis and secretion of the high molecular weight antigen, recognized by BST-1, VERO cells were infected with trypomastigotes (strain Y) and after 24, 48, 72, 96 and 144 hours, the parasites present in VERO cells and in the conditioned medium were collected. The parasite-free supernatant of conditioned medium was also analyzed. The number of trypomastigotes and amastigotes were determined and the BST-1 reactivity analyzed by Western Blot. It was observed that the level of 160-200kDa component recognized by BST-1 increases during infection indicating that amastigotes are able to synthesize the component of 160-200 kDa. On the other hand, the presence of the 160-200 kDa antigen in the parasite-free supernatant was detected after 96 hours of infection, when trypomastigotes start to be liberated from VERO cells to culture medium.

In infectivity assays it was verified that mAb BST-1 at a concentration of 2.5 µg/ml was able to inhibit about 70% of trypomastigotes infectivity of VERO cells. These results suggest that the epitope/antigen recognized by mAb BST-1 is involved in adhesion/internalization of trypomastigotes.

A better characterization of this trypomastigote high molecular weight component recognized by BST-1 and the mechanism of secretion are under study.

Supported by FAPESP and CNPq

BQ44 - PURIFICATION AND PARTIAL CHARACTERIZATION OF A FLAGELLAR GLYCOPROTEIN FROM *LEISHMANIA (VIANNIA) BRAZILIENSIS*

Yoneyama KAG, Takahashi HK, Straus AH

Department of Biochemistry, Universidade Federal de São Paulo, São Paulo, SP, Brazil

Glycoconjugates are usually involved in *Leishmania*-macrophage interactions. Studies carried out in our lab demonstrated the involvement of flagellar 180 kDa glycoprotein specific of *Leishmania (Viannia) braziliensis* in the interaction of promastigotes with macrophages during the infection (Silveira et al, Int J Parasitol., 31:1451-8, 2001). Promastigote forms of *L. (V.) braziliensis* (WHOM/BR/1987/M11272) were cultivated in LIT medium supplemented with 10% of heat inactivated fetal calf serum. The flagella were separated from promastigote bodies by gasose nitrogen cavitation (350 psi) and purified by sucrose gradients. The flagellar proteins were solubilized with n-Octyl glucoside 50mM, PMSF 0.2mM and EDTA 1mM in PBS. The solubilized proteins were diluted in sample buffer and polyacrylamide gel electrophoresis (SDS-PAGE) was performed. A high molecular weight flagellar glycoprotein was purified by SDS-PAGE. After gel staining with Coomassie blue the proteins were electroeluted. The purified 180 kDa flagellar glycoprotein was analyzed by SDS-PAGE and tested by Western blotting with 7 biotinylated lectins and with monoclonal antibody (mAb) SST-3, which is specific for this flagellar glycoprotein. *Helix pomatia* lectin and mAb SST-3 recognized only the 180 kDa flagellar glycoprotein while Concanavalin A bound to the 180 kDa glycoprotein and various other parasite glycoproteins. By indirect immunofluorescence carried out using SST-3 and biotinylated *Helix pomatia* lectin it was detected a positive fluorescence only in flagella. When promastigotes were pre-incubated with mAb SST-3 and than with biotinylated *H. pomatia* lectin and streptoavidin conjugated to fluorescein, no fluorescence was detected, indicating that mAb SST-3 blocked *H. pomatia* lectin binding to gp180, and that the this glycoprotein presents a-GalNAc residues in the glycan structure. Further purification of gp180 is required to improve its characterization by determination of the isoelectric point and NH_2-terminal region sequence.

Supported by: CAPES, FAPESP and CNPq

BQ45 - THE ROLE OF SULFATED GLYCOSAMINOGLYCANS IN THE INVASION OF CARDIOMYOCYTES BY *TRYPANOSOMA CRUZI* AND BIOCHEMICAL CHARACTERIZATION OF ITS RECEPTOR

Oliveira FOR Jr[1], Alves CR[2], Toma L[3], Nader HB, Côrtes LMC[2], Calvet CM[1], Almeida D[1], Azevedo-Pereira RL[2], Albuquerquje-Cunha JM[4], Meirelles MNL[1] & Pereira MCS[1]

[1]Departamento de Ultra-estrutura e Biologia Celular, [2]Departamento de Bioquímica e Biologia Molecular, IOC/ FIOCRUZ, RJ, Brasil. [3]Departamento de Bioquímica UNIFESP, SP & [4]Lab. de Biologia Celular e Tecidual, UENF, Campos, RJ.

The ability of *Trypanosoma cruzi* to recognize molecules on the surface of phagocytic and non-phagocytic cells is determinant to its survival within vertebrate host. Evidences of the participation of sulfated proteoglycans in the recognition process have been reported in many human pathogens, including *T. cruzi* via a heparin-binding protein (penetrin). Recently, we have demonstrated that heparan sulfate proteoglycan (HSPG) mediate the invasion of trypomastigotes in cardiomyocytes (Calvet et al., 2003). However, the structure of HSPG molecule implicated in the receptor-ligand binding and the role of others sulfated glycosaminoglycans (GAGs) in this interaction is not completely

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understood. Additionally, we were interested to isolate the *T. cruzi* surface receptor in order to verify its interaction with the mammalian cells and the peritrophic membrane of its vector, *Rhodnius prolixus*.

To test the effect of GAGs on *T. cruzi* invasion, culture-derived trypomastigotes, Dm28c clone, were pretreated with 20µg/ml of heparin, keratan sulfate (KS) or heparan sulfate (HS) fragments, which were obtained by enzymatic treatment. In the competition assays, the parasites were incubated for 2h at 37ºC with cardiomyocytes and the percentage of infection evaluated. Treatment of trypomastigotes with heparin decreased considerably the parasite invasion achieving an inhibition of 80% in the infection ratio. The HS fragment obtained by heparitinase II cleavage reduced the infection level, while KS displayed no inhibitory effect.

These parasites receptor was isolated by the association of the Triton X-114 method with heparin-sepharose and DEAE-cellulose chromatography. SDS-PAGE analysis of the parasites hydrophobic protein extracts revealed two distinct proteins bands, 65.8 kDa and 59.0 kDa, eluted from the heparin affinity chromatography, which were in the majority eluted at 0.5M and 1.0M of NaCl for trypomastigotes and epimastigotes, respectively. The radioactive analysis suggests that the surface receptor is 2.7-fold higher in trypomastigotes compared to levels found in epimastigote forms. Furthermore, similar molecular mass duplet was also detected in both parasite forms after the heparin or heparan sulfate recognition on the parasites proteins extract by Western blot. The anionic chromatography data show evidence that these two proteins exhibit similar charge net in both *T. cruzi* forms. These results also suggest that a homogeneous sample occur in the low salt concentration, 0.075M of NaCl. Further studies are in progress to verify the ability of these isolated proteins to recognize mammalian and the *R. prolixus* peritrophic proteins.

Supported by FIOCRUZ, FAPERJ and CNPq.

BQ46 - PRELIMINARY ANALYSIS OF VESICLE PROTEINS FROM *LEISHMANIA* SPECIES.

de Oliveira AHC[1], Rosa JC[2], Greene LJ[2], Ruiz, JC[1], Cruz AK[1] and Ward RJ[3].

[1.] Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos - FMRP-USP. [2.] Centro de Química de Proteínas- FMRP-USP. [3.] Departamento de Química - FFCLRP-USP, Ribeirão Preto, SP.

With the aim of identifying secreted proteins in both Old World and New World *Leishmania* species, intrecellular vesicles were extracted by centrifugation from cell lysates of promastigotes forms of *L. (L.) major* and *L. (L.) amazonensis*. After disruption of the vesicles by ultrasonication, soluble proteins were separated by ultracentrifugation, and were analysed by SDS-PAGE and 2D-PAGE. Protein spots were analysed by ESI-MS/MS mass spectometry of peptides generated by tryptic digestion, and the amino acid sequences of these peptides were compared with protein and DNA sequence databases using BLAST and FASTS. Preliminary results show that the protein extracts from both species includes the cell surface secreted proteins Gp46 and Gp63, which demonstrates the viability of whole vesicle extract proteomic analysis for the identifation of secreted proteins in trypanosomatid parasites. Furthermore, differences in the vesicle protein profiles were observed comparing extracts from *L. (L.) major* and *L. (L.) amazonensis*, which suggest that this methodology may be applied to a subcellular proteomic approach to study differential expression patterns of the parasite.

Supported by FAPESP, CNPq and PRONEX.

BQ47 - ANALYSIS OF THE PROTEOME OF *TRYPANOSOMA CRUZI* USING TWO-DIMENSIONAL GEL ELECTROPHORESIS AND MALDI TOF

Jaime Paba[1]; Jaime M. Santana[2], Antonio R. L. Teixeira[2]; Wagner Fontes.[1], Marcelo V. de Sousa. [1] and. Carlos A. O. Ricart[1,#]

[1] Brazilian Center for Protein Research, Department of Cell Biology, University of Brasilia, Brasília, 70910-900- DF, Brazil. [2] Laboratorio Multidisciplinar de Pesquisa em Doença de Chagas, University of Brasília, Brasilia, 70910-900, DF, Brazil.

Trypanosoma cruzi, the protozoan which causes Chagas disease, possesses a complex life cycle involving different developmental stages. Experimental conditions for two-dimensional electrophoresis (2-DE) analysis of *T. cruzi* trypomastigote, amastigote and epimastigote proteomes were optimized. Comparative proteome analysis of the life stages were done, revealing that few proteins included in the 2-DE maps displayed significant differential expression among the three developmental forms of the parasite. In order to identify landmark proteins, spots of the trypomastigote 2–DE map were subjected to MALDI-TOF MS peptide mass fingerprinting, resulting in 26 identifications that corresponded to 19 different proteins. Among the identified polypeptides there were heat shock proteins (chaperones, HSP 60, HSP 70 and HSP 90), elongation factors, glycolytic pathway enzymes (enolase, pyruvate kinase and 2,3 bisphosphoglycerate mutase) and structural proteins (KMP 11, tubulin and paraflagellar rod components). The relative expression of the identified proteins in the 2-DE maps of the three *T. cruzi* developmental stages is also presented.

Supported by CNPq, CAPES, UnB

BQ48 - DIFFERENTIAL PROTEIN EXPRESSION PROFILE IN HEARTS OF CHRONIC CHAGAS'DISEASE OR IDIOPATHIC DILATED CARDIOMYOPATHY

Teixeira, P. C.; Iwai, L. K.; Kalil, J.; Cunha-Neto, E.

Laboratório de Imunologia, Instituto do Coração - Incor, Hospital das Clínicas, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP AND Instituto de Investigação em Imunologia, Institutos do Milênio, Brazil

Chronic Chagas'disease cardiomyopathy (CCC) is an often fatal outcome of *Trypanosoma cruzi* infection, having shorter survival than idiopathic dilated cardiomyopathy (IDC), a clinically similar cardiomyopathy showing less inflammatory phenomena. In order to analyze the molecular changes in the affected myocardium in response to chronic inflammation in CCC, we used proteomic analysis to compare the protein expression profile in heart samples from CCC and IDC patients and identify differentially expressed proteins. Myocardial homogenates of heart samples from end-stage CCC and IDC hearts explanted during heart transplantation were subjected to bidimensional electrophoresis (3-10 pI gradient and 12% polyacrylamide gel electrophoresis). Differentially expressed Coomassie blue-stained protein spots were selected with imaging software; selected spots in gels were excised, subjected to tryptic digestion and processing in a robotic workstation. Protein identification was performed by peptide mass fingerprinting with the aid of MALDI-ToF mass spectrometry and virtual tryptic digestion of proteins in sequence databanks. Results have shown more than 200 Coomassie blue-stained spots in each gel. Preliminary analysis has identified mitochondrial creatine kinase as the protein with most significantly reduced expression in the myocardium of a CCC patient, as compared to a paired IDC patient. Given the fundamental role of mitochondrial creatine kinase in the translocation of high-energy phosphate from the mitochondrion to generate cytoplasmic ATP, this finding may be related to the shorter survival observed in CCC as compared with IDC.