07 FUNGAL CELL BIOLOGY

07.001 - CALCINEURIN CONTROL OF TEMPERATURE-INDUCED DIMORPHISM AND GROWTH OF *PARACOCCIDIOIDES BRASILIENSIS*: ROLE OF LIPID PEROXIDATION.

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Introduction and Objectives: *Paracoccidioides brasiliensis* is a dimorphic fungus that causes paracoccidioidomycosis, a systemic mycosis prevalent in Latin America with most cases found in Brazil. The dimorphism from mycelium to yeast cell is triggered by temperature rise from 25ºC to the human body temperature of 37ºC and is necessary for infection, expression of virulence factors and pathogenicity. Despite its importance, very little is known about the intracellular mechanisms that control dimorphism in this fungus. In this work, we investigated whether calcineurin, a Ca^{2+}/calmodulin-activated serine/threonine phosphatase, is part of the intracellular mechanism that control dimorphism, growth and viability of *P. brasiliensis* under environmental temperature and mammalian host temperature. **Methods:** *P. brasiliensis* Pb18 isolate was cultured in liquid YPD medium at either 25ºC (mycelium) or 37ºC (yeast) for 7 days, then diluted H"10x in new medium and cultured for two additional days at the same temperature, when the drugs were added to the culture. For dimorphism studies, drugs were added to the culture 1 hour before the temperature shift. The fungus was further cultured for 8-10 days in the presence of the drugs. **Results:** The calcineurin inhibitor cyclosporin A (CsA) at 5 mg/ml blocked *P. brasiliensis* dimorphism and the subsequent growth of the fungus at 37ºC. Growth of yeast phase cells at 37ºC was also blocked by calcineurin inhibition. Mycelium growth at 25ºC was, however, not affected. *P. brasiliensis* cultured with CsA under the different conditions studied remain metabolically active, suggesting that calcineurin did not control the viability of the fungus. We also suggest that the temperature rise leads to a heat stress with generation of reactive oxygen species (ROS) that could negatively affect dimorphism and growth. In fact, butilated hydroxytoluene (BHT-20 μM), that prevents lipid peroxidation, slightly increase the growth of the fungus after temperature shift. However, BHT did not change CsA effect on either growth or dimorphism. **Conclusion:** The temperature shift that triggers mycelium to yeast transition of *Paracoccidioides brasiliensis* leads to the activation of calcineurin, which is required for both dimorphism and the following growth of the fungus, as well as the growth of yeast cells. Under our culture conditions, growth of mycelium and viability of the fungus at different phases occurred independently on calcineurin. We also suggest some growth inhibition by ROS during dimorphism and the subsequent growth of the fungus and that dimorphism blockage induced by inhibition of calcineurin does not induce lipid peroxidation. **Financial support:** Fapesp, CNPq.

07.002 - PROSTAGLANDIN E_2 PRODUCTION BY HIGH AND LOW VIRULENT STRAINS OF *PARACOCCIDIÒIDES BRASILIENSIS*

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Introduction and Objectives: Enhanced prostaglandin production during fungal infections could be an important factor promoting fungal growth and consequently chronic infection. Host cells could be one source of these eicosanoids during fungal infection. However another potential source of them is the fungal itself. Some works have been demonstrated that some fungi such as *Cryptococcus neoformans* and *Candida albicans* produce and secrete prostaglandins into its surroundings without an exogenous source of arachidonic acid. However, there are no works showing that *P. brasiliensis* could utilize exogenous or endogenous sources of this component to produce prostaglandins. Our objective was to assess if *P. brasiliensis* produce PGE_2 and if this production was related with its survival *in vitro.* **Methods and Results:** *P. brasiliensis* strains 265 and 18 were grown in GPY culture medium at 37ºC for 6 days. After, the cultures were harvested and a yeast suspension of $4x10^4$ viable fungi/mL were incubated in RPMI medium by an additional period of 4h in the absence or presence of different concentrations of indomethacin (10, 5, 2.5, 1 and 0.5 mg/mL), and prostaglandins production measured using specific competitive ELISA kit. Moreover before and after indomethacin treatment the recovery of colony-forming units (CFU) were evaluated by fungal plating in BHI agar medium. Both strains of *P. brasiliensis* produce high concentrations of PGE_2 (mean±sem Pb18= 249±7,5pg/mL and Pb265= 242±3,8 pg/mL). PGE_2 levels were significantly reduced after indomethacin treatment (Pb18= 130±5,5; 102±28; 141±13; 131±37,5 and 204±7,2. Pb265= 108±4,4; 110±5,7; 103±28; 106±19,6 and 153±15,6 pg/mL, for the concentrations of 10, 5, 2.5, 1 and 0.5 mg/mL respectively). Moreover, the indomethacin treatment inhibited the CFU recovery of both strains (CFU Pb18 alone= 275±81; CFU Pb18+INDO= 175±69; 161±76; 163±75; 159±87 and 166±88. CFU Pb265 alone= 160±33; CFU Pb265+INDO= 82±15; 125±5; 122±18; 120±16 and 153±19, for the concentrations of 10, 5, 2.5, 1 and 0.5 mg/mL respectively). **Conclusion:** The results suggest that high and low virulent strains of *P. brasiliensis* produce high levels of prostaglandins that promote fungal growth, revealing a virulence mechanism that has potentially great implications for understanding the mechanisms of chronic fungal infection.

07.003 - NEW INSIGHTS INTO THE CELL CYCLE PROFILE OF *PARACOCCIDIOIDES BRASILIENSIS*

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Introduction and Objectives: The control mechanisms that regulate cellular and nuclear division during cell growth of the pathogenic fungus *Paracoccidioides brasiliensis* are still poorly understood. Considering the multiple budding cellular division and multinucleate nature of this fungus, one would expect a strict regulation of the cell cycle and the interaction between DNA replication, nuclei segregation and budding. The main goal of this work was to study nuclear and/or cellular division of *P. brasiliensis* yeast cells, focusing on the analysis of cell cycle progression under different environmental conditions. **Methods and Results:** In order to achieve the proposed objectives, we optimized a flow cytometric technique for the evaluation of *P. brasiliensis* yeast cells cycle profile based on nuclear DNA content, using SYBR Green I staining. Exponentially growing cells in defined or complex nutritional environments showed an increased percentage of daughter cells in accordance with the fungus´ characteristic multiple budding and high growth rate. However, during the transition to the stationary phase progression of cell cycle differed according to the culture medium. In complex medium, an accumulation of cells with higher DNA content or pseudohyphae-like structures was observed, whereas in defined medium arrested cells mainly displayed two distinct DNA contents in contrast to what is usually observed for other microbial populations. Furthermore, the fungicide benomyl, a drug that disturbs microtubule assembly, induced an arrest of the cell cycle with accumulation of cells presenting high and varying DNA contents, consistent with this fungus' unique pattern of cellular division. **Conclusion:** Altogether, our findings indicate that a set of DNA replications occur prior to multinuclear segregation and multiple budding. These data also suggest that in *P. brasiliensis* yeast cells the mitotic cell cycle regulation of may follow control mechanisms different from those previously described for other biological systems. Furthermore, the study of the cell cycle profile of pathogenic fungi (such as *P. brasiliensis*) may be relevant from a clinical perspective, namely, the assessment of the activity of antifungal drugs targeting key molecules involved in the regulation of cell cycle progression. **Financial support:** Almeida, A. J. was supported by a fellowship from Fundação para a Ciência e Tecnologia (FCT), Portugal (contract SFRH/BD/8655/2002). This work was supported by a research grant from FCT (Grant Number: POCTI/ESP/45327/2002).

07.004 - DETECTION OF EXOCELLULAR PROTEASES FROM *PARACOCCIDIOIDES BRASILIENSIS*

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Introduction and objectives: *Paracoccidioides brasiliensis* is the causative agent of paracoccidioidomycosis (PCM), a chronic granulomatous disease of mucous membranes, skin, and pulmonary system. Natural infection is presumed to be via the respiratory route. Many proteins represent virulence factors for these fungal organisms, some are involved in the fungus-host interaction, while others are involved in tissue invasion like an exocellular serine-thiol protease enzyme (Med Mycol. 38 Suppl 1:79-86, 2000). The aim of the present study is evaluate others exocellular proteases in the culture filtrates of *P. brasiliensis*. **Methods and Results:** The components of *P. brasiliensis* (Pb18 strain) culture filtrates were separated by gel filtration chromatography in a TSK-G3000sw column (30 cm x 0.8 cm). The chromatographic fractions containing protease activity were pooled and submitted to Ion-exchange chromatography in a Mono Q column. At present, 3 fractions with enzymatic activity were identified. So, the peaks with protease activity were analyzed by SDS-PAGE electrophoresis and inhibition enzymatic assays were carried out by using PMSF, Leupeptin, EST and EDTA. The highest activity was achieved with the P1 fraction that presented specific activity of 1179 U/mg, which was inhibited by PMSF (43% inhibition). On the other hand, the P2 fraction had its specific activity of 257 U/mg inhibited only after being treated with EDTA, which provides an inhibition level of approximately 55%. The last fraction analyzed, P3, presented specific activity of 167 U/ mg. The level of inhibition of P3 activity was assayed providing 70% inhibition with PMSF and 50% with EDTA. Leupeptin and EST had no effect on the inhibition of these fractions.**Conclusion:** Since the molecular characterization of these proteases will enable us to better understand its role as possible virulence factors as well as their evolvement in fungal tissue invasion, additional *P. brasiliensis* fractions have been analyzed in order to identify new proteases. **Financial support:** CAPES, FAEPA

07.005 - SIDEROPHORE PRODUCTION IN *PARACOCCIDIOIDES BRASILIENSIS*

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Introduction and Objectives: Iron is an essential nutrient required for the metabolism and growth of nearly all living organisms. Although iron is the second most abundant metal on earth (after aluminum), it is generally present in very insoluble compounds (oxides-hydroxides) in aerobic environments that are not readily bioavailable. Fungi overcome the problem of obtaining iron in a variety ways, being the uptake of iron by secretion of low-molecular-mass iron chelators called siderophores the most common (Annu. Rev. *Biochem. 50:* 715-731, 1981). Siderophores strongly bind Fe^{3+} and deliver the iron to the microbe via high affinity internal uptake systems (Infect. Immun. v.72, p.1402-1408, 2004). Here we describe the identification of siderophore production in five isolates (Pb18, Pb339, PbAP, Pb1925 and Pb608) of *P. brasiliensis.* Although it had already been demonstrated that *P. brasiliensis* produces growth enhancing factors present in the culture filtrate and that some siderophores produced by other fungi can be substituted for this culture filtrate (J Med Vet Mycol. v.26, p.351-358, 1988) the identification and biochemical characterization of siderophore production in *P. brasiliensis* is wanted. **Methods and Results:** The siderophore identification in *P. brasiliensis* was done using the universal chemical CAS assay (Analytical Biochem. 160: 47-59, 1987). *P. brasiliensis* strains were grown in a modified MM9 medium. We identified siderophore production in all (five) isolates tested. The siderophore identification assays were done either on solid or liquid medium. Isolate Pb608 seems to be the major siderophore producer by the method used. The isolation, purification and characterization of the siderophore structure of *P. brasiliensis* are still in progress. **Conclusion:** As limitation of iron availability is utilized by many animal species as an antimicrobial defence strategy we propose that siderophore production in *P. brasiliensis* is a fundamental mechanism of virulence for this organism, as well as a promissory target for drugs. As siderophores are immunogenic compounds it is also of great interest for vaccine development since there is no evidence of siderophore production on its host. **Financial support:** FAPESP

07.006 - EFFECT OF JBE (JACALIN BINDING EXOANTIGEN) FROM *PARACOCCIDIOIDES BRASILIENSIS* ON PHAGOCYTOSIS BY MURINE PERITONEAL MACROPHAGES

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Introduction and Objective: The nature of interaction of *Paracoccidioides brasiliensis* with macrophages is critical for establishment of the fungal infection. In the present study, the effect of JBE (Jacalin Binding Exoantigen) from *P. brasiliensis* in attachment and phagocytosis by murine peritoneal macrophages was investigated. **Methods and Results:** JBE was obtained from yeast cells culture supernatants purified by affinity to jacalin, and revealed, by SDS-PAGE, as a high molecular mass component (HMMC, ~190-kDa) and a 70-kDa band (paracoccin). To phagocytosis assay, macrophages treated or not with JBE (100 µg/ml) were incubated for 24hs with yeasts*FITC treated or not with N-Acetyl glucosamine (GlcNAc) or anti-JBE Fab. An average of 200 macrophages was counted to determine the phagocytic indexes (PI). JBE was able to promote inhibition of phagocytosis at 41% by JBE-treated macrophages (control PI = 273 ± 5,84; JBE-treated macrophages PI = 163,56 ± 6,25). The phagocytosis inhibition increased to 64% when JBE-treated macrophages were incubated with yeast cells treated with GlcNAc (PI = 98,50 ± 13,67). Anti-JBE Fab was able to inhibit the phagocytosis at 30% (PI =197,96 ± 12,47) and both JBE-treated macrophages and yeast cells pre-treated with anti-JBE Fab increased phagocytosis inhibition to 62% (PI = 105,73 ± 5,11). **Conclusion:** These results suggest that JBE fraction has component able to promote invasion of fungus and could be involved on the fungus and host cell interaction. **Financial support:** CAPES/FAPEMIG.

07.007 - THE GLYCERALDEHYDE-3- PHOSPHATE DEHYDROGENASE OF *PARACOCCIDIOIDES BRASILIENSIS* IS A CELL SURFACE PROTEIN AND IS RELATED TO THE FUNGUS ADHESION

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Introduction and Objectives Paracoccidioidomycosis (PCM) is a systemic mycosis affecting humans that is geographically confined to Latin America. The disease is caused by *Paracoccidioides brasiliensis*, a dimorphic fungus that undergoes a complex differentiation *in vivo*. The ability of the pathogen to interact with the host superficial structures is essential for its virulence. Glyceraldehyde -3-phosphate dehydrogenase (GAPDH) has been characterized as an adhesin in several pathogens. The complete cDNA encoding the GAPDH of *P. brasiliensis* was overexpressed in an *Escherichia coli* host to produce high levels of the recombinant protein. In this work we analyzed the cellular localization of the GAPDH, as well as its role in the interactions between *P. brasiliensis* and in vitro cultured cells. **Methods and Results** Western blot experiments and immunoelectron microscopy studies demonstrated that the GAPDH is localized intracelulary as well as in the cell wall of *P. brasiliensis.* We investigated the GAPDH role as an adhesin molecule. GAPDH presents the ability to bind to host proteins of the extracellular matrix (fibronectin, laminin and collagen type I) as showed by affinity ligand assays. To evaluate the role of GAPDH in the adhesion and invasion process of infection we performed experiments with *in vitro* cultured pneumocytes A549 cells. The presence of the recombinant GAPDH, as well as, anti-GAPDH antibody, promoted inhibition of adhesion and invasion of *P. brasiliensis* to the *in vitro* cultured cells. **Conclusion** The results indicate that the GAPDH from *P. brasiliensis* is a cell wall protein, that presents ability to bind to extracellular matrix and function as an adhesin molecule related to the fungus pathogenesis. **Financial support:** CAPES and MCT/CNPq

07.008 - IDENTIFICATION AND PARTIAL CHARACTERIZATION OF A FETUIN BINDING PROTEIN FROM *PARACOCCIDIOIDES BRASILIENSIS*

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Introduction and Objectives: Significant advances have been made in our understanding of the adhesion of pathogenic microorganisms to host tissues, the initial colonization and further dissemination. Over the past few years, several fungal adhesins have been discovered, among them, an adhesin derived from *Penicillium marneffei* conidia that bind to extracellular matrix glycoproteins, laminin and fribronectin, via a sialic acid-dependent process (Inf. Immunity, 67(10): 5200–5205, 1999). This sialic acid-dependent interaction can be important in mediating attachment of a variety of fungus to both host tissues and cells. Thus, since *Paracoccidioides brasiliensis* derived components interact with host cells, a property that might be evolved in the paracoccidioidomycosis (PCM) pathogenesis, we aimed to report a *P. brasiliensis* component that interacts with BALB/c mice lung extract. **Methods and Results:** *P. brasiliensis* yeast cells of BAT isolate were maintained in liquid F-12 medium and then treated with thimerosal and protease inhibitor. BAT antigens obtained by sonication and centrifugation at 10000 x g were submitted by affinity chromatography in a sepharose-fetuin column. The bound material eluted with 1M NaCl was dialyzed against water by ultra filtration and its protein content was estimated using a BCA Kit. The gradient 5.0-20% SDS-PAGE of fetuin binding protein performed in reducing conditions show a predominant band with apparent MM 35 kDa. The fetuin binding protein were blotted onto a nitrocellulose membrane, assayed with biotinalated lung extract and revealed with alkaline phosphatase. Either BAT antigens or fetuin binding proteins were recognized by the lung extract. An isoelectric focusing, as well as carbohydrate stain, mass spectrometric analyses and biological assays are in progress in order to better characterize this protein. **Conclusion:** Accordingly, we are presently attempting to purify this BAT antigen as a first step in its full characterization. Considerably more data are required to actually implicate this process in pathogenesis. **Financial support:** FAPESP, FAEPA

07.009 - VARIATION ON MYCELIAL-TO-YEAST PHASE TRANSITION AND PRODUCTION OF CONIDIA IN DIFFERENT ISOLATES OF *PARACOCCIDIOIDES BRASILIENSIS* OBTAINED FROM HUMAN, ARMADILLO AND DOG

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Introduction and objectives: The dimorphic fungus, *Paracoccidioides brasiliensis*, is the etiological agent of paracoccidioidomycosis (PCM), an important systemic mycosis in Latin America. Several pieces of evidence point to soil as its saprobe habitat, where it produces assexual infecting spores, the conidia. Dimorphism is an important virulence factor, since the mycelial-to-yeast (M-L) transition has been considered essential to its establishment and dissemination in the hosts' tissues. Therefore, more comparative studies about this transition as well as production of conidia are indispensable to improve knowledge of the fungus´s biology. This work aimed to evaluate the production of conidia and the temperature M-L transition in different isolates. **Methods and results:** Nine isolates were used, four from armadillos (T4, T10, T13 and S1), four from human patients (Bt84, Bt85, Pb265 and D01) and one recently isolated from a dog (Pb-dog). Mycelial fragments of each isolate were cultured on GPY (Glucose, Peptone Yeast Extract Agar) in a Petri dish and in a slide culture for both macroscopic and microscopic analyses of the dimorphism at different temperatures (30, 32, 34 and 36°C). The time necessary for the transition was estimated by culturing mycelial fragments (grown at 25°C) on GPY at 36°C followed by macroscopic observation every four days. The slides were stained with Lactophenol-cotton-blue, the dimorphism and presence of conidia were registered. The isolate T4 was the first to begin the transition, at 30°C. The isolates T10 and Bt84 showed yeast forms only at 34°C. The other isolates began the transformation at 32°C. The isolates that showed a higher transition temperature required more time to convert to yeast form when the mycelial form was cultured directly at 36°C. The production of arthroconidia, was observed at 30°C in T4, T13, S1, Bt85 and Pb-dog, at different levels of sporulation. The isolates Bt85 and S1 showed a higher production of arthroconidia (about 10 per field). **Conclusions:** The unexpected production of arthroconidia under conditions of 30°C in a rich medium indicates that the sporulation is not so rare, but it might be very often in certain isolates. As observed in other studies carried out in our lab, the sporulation is higher on soil and the number of arthroconidia from different isolates in this substrate is proportional to that observed on GPY medium. These preliminary results also confirmed the phenotypic variability concerning dimorphism. As documented by several authors, although this feature is influenced by external factors, it has a genetic control and this genotype variation should be studied further. As observed in *Histoplasma capsulatum*, low levels of heat shock protein (HSP) gene are correlated with thermosensitivity and low virulence. Studies that correlate these data with virulence and differential expression of HSP gene are underway in our lab. **Financial support:** FAPESP (grants 02/00466-5 and 04/12949-6)

07.010 - SEXUAL REPRODUCTION OF *PARACOCCIDIODES BRASILIENSIS* IN ITS PARASITIC FORM

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Introduction and Objectives -The Ascomycota are characterized by bearing their sexual spores within an ascus. The ascus is a cell that at first contains a diploid nucleus resulting from karyogamy, and this nucleus undergoes meiosis. Haploid ascospores with thick walls are formed within the ascus. In most of these fungi, the asci are borne in or upon a sporocard, the ascoma. *Paracoccidiodes brasiliensis*, the ethiological agent of paracoccidioidomycosis, was first included in the Kingdom Fungi: Form-Division Deuteromycota. In 1994, by phylogenic studies based on large subunit ribosomal RNA sequence comparisons it was identified as a member of Division Ascomycota. With respect to asexual reproduction, as a yeast form, in infected tissues or at 37°C in artificial media, multi-budding and usually multi-nucleate yeast cells of various shapes and sizes are formed. In contrast, for many years the mycelial form of this dimorphic fungus have been considered to be a sterile mold as far as demonstration that under appropriate growth medium cultured at room temperature this mycelium could sporulate profusely. But never the sexual form of this pathogen involved in its cycle life were described. The aim of this work is to show these forms. **Methods:** For determine the reproduction sexual cycle of this fungus many infections experiments of HeLa and Vero culture cells by yeast forms of this fungus were developed. The infected cultures were revealed by different coloring and antigen-antibody reaction at different periods post-infection and analyzed in different microscopes. **Results:** Comparing and analyzing hundreds of photographs of infected cells by the fungus, after many revelations observed in many microscopes, we present in this work sequential forms of *Paracoccidioides brasiliensis* that correspond to stages of the sexual reproduction in the complex life cycle of this pathogen. **Conclusion:** In this process, two blastoconidias expressing many buds by plasmogamia and karyogamia form the zygote that secretes an extra glycoprotein matrix, corresponding to an ascus, containing multiple small and cell wall less ascospores, characterizing a probable extended dikaryotic yeast phase. At the first minutes post-infection yeast cells of *P. brasiliensis* trigged by stress enviromental host relationship show an explosive production of blastoconidias expressing many buds in the infected cultures; an intense secrection of the ascus fibrillar matriz and actin polymerization of their cytoskeleton. All manifestations that attest to the resourceful nature of an eukaryote. **Financial support:** FAPESP, FAEP/UNICAMP, CAPES

07.012 - CELL WALL LOCALIZATION IN *PARACOCCIDIOIDES BRASILIENSIS* OF THE MITOCHONDRIAL HEAT SHOCK PROTEIN MDJ1, A DNAJ HOMOLOGUE.

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Introduction and Objectives: We have characterized an *MDJ1*-homologue gene of *P. brasiliensis* (*PbMDJ1*). In eukaryotic cells, Mdj1 is the mitochondrial member of the DnaJ family of chaperones, which function alone or in cooperation with the DnaK (hsp70) complex. The aim of this work was to characterize PbMdj1 in terms of cellular localization and biological role. **Methods and Results**: We have expressed a truncated form of PbMdj1 containing the N-terminal region (rPbMdj1) and used it to generate anti-rPbMdj1 antibodies of high immunoblotting titers. These antibodies recognized a mitochondrial heat shock component of an estimated M_r of 55 kDa, which is compatible with the deduced size of PbMdj1 devoid of the predicted mitochondrial target. We used these antibodies to investigate the cellular localization of PbMdj1 in *P. brasiliensis*. By transmission electron microscopy and immunogold label, we detected gold particles distributed in the mitochondria, however intense labeling in the cell wall was also observed. In this compartment, the gold particles are apparently more concentrated in the inner layer, but they are also abundantly present in the budding region. Labeling with pre-immune serum was scarce. Our preparations showed a large number of mitochondria near the cell membrane, yet not in contact with it. Surface labeling of *P. brasiliensis* yeast cells with anti-PbMdj1 (and not with pre-immune serum) was additionally demonstrated by confocal fluorescence microscopy and by flow citometry (FACS) analysis. By FACS, we observed a typical dose-response curve upon incubation of fixed cells with 50, 100 or 200 mg/mL of total anti-rPbMdj1 IgG. At 200 mg/mL, over 50% of the cells were labeled, against 7% of the cells that were nonspecifically recognized by the pre-immune IgG. Using alkaline solution added with b-mercaptoethanol, we obtained a mild cell wall protein extract for immunoblotting assays with anti-rPbMdj1. A 55-kDa component, comparable with the mitochondrial band recognized by anti-rPbMdj1, was specifically detected, suggesting that the component responsible for cell wall labeling of *P. brasiliensis* is the same as that found in mitochondria. Moreover, this result suggests that the molecule was first processed in this organelle before localizing to the cell wall. Although sera from patients suffering from PCM could recognize recombinant PbMdj1, polyclonal anti-rPbMdj1 IgG caused no effect on *P. brasiliensis* growth in vitro. **Discussion:** The results presented here show for the first time the presence of a DnaJ homologue in a fungal cell wall and open the possibility for a better understanding of the role of heat shock proteins in this extra-mitochondrial localization. Although anti-rPbMdj1 apparently did not perturb *P. brasiliensis* growth *in vitro*, an immunological modulatory role of PbMdj1 cannot be discarded due to the presence of a potential T-cell receptor in its structure. **Financial support:** FAPESP and CNPq

07.013 - EFFECT OF GP43 AND 30 KDA ADHESINS OF *PARACOCCIDIOIDES BRASILIENSIS* IN THE CYTOSKELETAL DISARRANGMENT AND APOPTOSIS IN EPITHELIAL CELLS

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Introduction and Objectives: Microbial virulence is a set of mechanisms that enable the infectious agent to penetrate the host protection barriers and then survive against the defense mechanisms, multiply and cause disease. Paracoccidioidomycosis presents a variety of clinical manifestations, and the fungus *Paracoccidioides brasiliensis* can reach many tissues, most importantly the lungs. Understanding of the mechanisms of dissemination is based on indirect evidence, and the polymorphic aspects of disease suggest that several virulence mechanisms are involved. The virulence of *Paracoccidioides brasiliensis* can be attenuated or lost after long periods of repeated subculturing and reestablished after animal inoculation. Only one adhesin (gp43) has been described until now, among the various identified components of *P. brasiliensis*, and gp43 shows adhesion to laminin. In previous studies a protein of 30 kDa, pI 4.9 was more evident in the protein extract of *P. brasiliensis* sample with a greater capacity to adhere and to invade the epithelial cells and had properties of adhesin. Laminin**,** but none of the other extracellular matrix components (ECM), such as fibronectin, collagen I and IV, bound specifically to the *P.brasiliensis* 30 kDa protein. The roles of 30 kDa and gp43 in cellular interactions were investigated and the adhesion of *P. brasiliensis* yeast cells was intensively inhibited by pre-treatment of epithelial cells with 30 kDa protein and gp43. For this reason, interactions between the fungal adhesins (gp43 and 30kDa) and A_{549} cells were evaluated, with the emphasis on cytoskeletal alterations during the interaction process fungus-cells. **Methods and Results:** Actin and cytokeratin could play a role in the *P. brasiliensis* adhesins interaction with the cells. Disarrangement and disruption of these filaments were observed after longer times of adhesin-cell contact. Differences in the pattern of cytoskeletal disarrangement had been demonstrated when compared the two adhesins. Thus, it seems that these proteins could be a ligands for some cytoskeleton components and may be candidate invasins. Using the TUNEL with fluorescent probe technique to label cells undergoing DNA fragmentation, it was shown that *P.brasiliensis* adhesins induces apoptosis in treated cells. Nuclear fragmentation and characteristic apoptotic cells were observed after 24 hours of contact between the adhesins and epithelial cells. Using

Bak and Bcl-2 antibodies, the cells treated with 30 kDa adhesin in initial periods (5 and 24 hours) expressed in similar way the two proteins and after 48 hours, increased expression of Bak occurred, that is a pro-apoptotic protein. When the cells were treated with the gp43 adhesin alterations in the expression of Bak and Bcl-2 had not occurred, thus demonstrating that the two adhesins induce apoptosis for distinct mechanisms. **Conclusions:** The adhesion and invasion of epithelial cells by *P.brasiliensis* may represent strategies employed to thwart the host immune response, and may help in the dissemination of the pathogen. **Financial support:** FAPESP, CNPq and PADC-FCF-UNESP.

07.014 - ISOLATION AND PARTIAL CHARACTERIZATION OF EXOSOME-LIKE VESICLES SHED BY *PARACOCCIDIOIDES BRASILIENSIS*

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Introduction and Objectives: *Paracoccidioides brasiliensis* is the dimorphic fungus responsible for paracoccidioidomycosis (PCM) in humans. We have characterized an exocellular thiol-dependent serine proteinase, which is secreted by the fungal yeast pathogenic form. This proteinase has a possible role in fungal dissemination because it is capable of *in vitro* selective degradation of proteins associated with the basal membrane, like laminin, fibronectin, type IV collagen and proteoglycans (Med. Mycol. 36:345-348, 1998). An interesting finding is that the proteinase activity against synthetic peptides is modulated by a high-molecular-weight polysaccharide complex secreted by the fungus (Microbes Infect., in press). We speculated that the serine-thiol proteinase could be shed to the extracellular medium inside vesicles, since occasionally an increase of activity was detected after freezing and thawing of culture supernatants. In some organisms, vesicular structures, known as exosomes, are derived from the exocytic fusion of multivesicular endosomes with the cell surface. Previous studies have supported the view that exosomes perform an acellular mode of communication and intercellular transfer of effector molecules. Exosomes have originally been isolated from several types of mammalian cells, and more recently from pathogenic microorganisms like *Trypanosoma cruzi* (Almeida et al., unpublished data). **Methods and Results:** Supernatant fluids from *P. brasiliensis* yeast cells cultivated in minimum medium enriched with fetal calf serum were filtered and concentrated in an Amicon system. Half of the material was ultracentrifuged and analyzed by electronic microscopy, where we could observe membranous vesicles sizing from 20 to 100 nm, not seen in preparations with medium alone. The remaining material was fractionated by gel-filtration chromatography. The eluted fractions were analyzed by ELISA with a reactive serum from a PCM patient. We observed that the high-molecular-weight peak fractions corresponding to the elution of vesicular structures were reactive with the PCM serum, suggesting the presence of *P. brasiliensis* antigens. Controls with sera from healthy individuals and fractionated medium alone were not reactive. Unfortunately, the supernatant fluids analyzed did not present the serine-thiol proteolytic activity, and consequentely neither did the vesicle preparations. We are currently performing a proteomic analysis of the vesicle fractions. **Conclusion:** Here we describe the isolation of exosome-like vesicular structures for the first time in a fungus. Exosomes have been recognized as important structures related with virulence of microorganisms and immunomodulation of the host. The finding of such components in a pathogenic fungus opens new perspectives to the study of the host-parasite relationship in PCM. **Financial support:** FAPESP, CNPq and BBRC/Biology/UTEP (NIH # 5G12RR008124)

07.015 - INDUCTION OF APOPTOSIS IN EPITHELIAL CELLS BY *PARACOCCIDIOIDES BRASILIENSIS* AND SOME COMPONENTS

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Introduction and Objectives: Paracoccidioidomycosis presents a variety of clinical manifestations, and the fungus *Paracoccidioides brasiliensis* can reach many tissues, most importantly the lungs. Understanding of the mechanisms of dissemination is based on indirect evidence, and the polymorphic aspects of disease suggest that several virulence mechanisms are involved. The ability of the pathogen to interact with the host superficial structures is essential to its virulence, but little is known about interactions between host cells and *P. brasiliensis* and their components. In previous studies we observed the kinetics of interaction with epithelial cells, and adhesion occurred after 30 minutes of contact between the epithelial cells and the Pb yeast. Pathogenic microorganisms utilize a variety of molecular strategies that subvert host cell mechanisms and enable these pathogens to invade susceptible host cells. Another consequence of invasion by intracellular pathogens is apoptosis or programmed cell death of the host cell, which is observed in professional or non-professional phagocytic cells. The ability of pathogens to induce apoptosis of phagocytes might be an important virulence factor, for it would curtail the host's defense mechanisms. *P. brasiliensis* and other fungi can exploit this approach to their own advantage, and their intracellular residence in epithelial cells could potentially elicit this type of cell death response. Therefore, we studied the interaction between *P. brasiliensis* and epithelial cells, with particular emphasis on the molecular events leading to apoptosis. **Methods and Results:** The apoptosis induced by *P. brasiliensis* in infected epithelial cells was demonstrated by various techniques: TUNEL, DNA fragmentation, Bak and Bcl-2 immunocytochemical expression and was quantified by flow cytometry. Using the TUNEL with fluorescent probe technique to label cells undergoing DNA fragmentation, it was shown that *P. brasiliensis* induces apoptosis in infected cells but not in uninfected ones. Nuclear fragmentation appeared after one hour and characteristic apoptotic cells were observed after two hours of contact between fungus and cells. After five hours the apoptotic bodies and rare yeast were found. Through molecular biology technique, it could be observed DNA fragments around 180bp of *P. brasiliensis* infected cells, characteristic of apoptosis. Using Bak and Bcl-2 antibodies, the infected cell in initial periods expressed in similar way the two proteins and after 24 hours, increased expression of Bak occurred, that is a pro-apoptotic protein, indicating the loss of competition between death and survival signals. To quantify apoptotic cells after infection with *P. brasiliensis* and its components, flow cytometry was used. It could be perceived that the number of apoptotic cells was proportional to the concentration and time of contact with *P. brasiliensis* components. **Conclusions:** The mechanisms of invasion of host cells, persistence within them, and subsequent induction of apoptosis of such cells may explain the efficient dissemination of *P. brasiliensis.* **Financial support:** FAPESP, CNPq and PADC-FCF-UNESP.

07.016 - IDENTIFICATION, PURIFICATION AND CHARACTERIZATION OF PROTEINS OF THE PROTECTIVE FRACTION (F0) OF *PARACOCCIDIOIDES BRASILIENSIS*

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Introduction and Objectives: Soluble antigens of *Paracoccidioides brasiliensis* yeast cells (PbAg) were fractionated in a fast protein liquid chromatography (FPLC) system, using Q-Sepharose anion-exchange resin. The FPLC system was able to resolve seven fractions, enumerated from 0 to VI, according to the elution on the NaCl gradient. The F0 fraction was able to elicit protective immunity in BALB/c mice in experimental PCM. In this work we analyzed the reactivity of sera from human with paracoocidioidomycosis in order to identify proteins in F0 that are responsible for the protective immunity. **Methods and Results:** The F0 fraction was sub-fractionated in FPLC system, using Mono-S anion-exchange resin and subsequently characterized about its protein profile through SDS-PAGE and Western blot. The peaks were eluted by 20 mM citrate buffer solution (pH 4.6) with an increasing gradient up to 1 M NaCl. We identified three sub-fractions designated 1 to 3. The protein analysis on SDS-PAGE showed less complex profile when compared with the original fraction. The protein content displayed bands with molecular mass ranging from 18 to 81 kDa. Sera from PCM patients recognized three main proteins localized on sub-fraction 2 with molecular mass of 27, 43 and 81 Kda. The N-terminal amino acid sequences comparison analysis of these proteins in the nonredundant GenBank at NCBI revealed that the protein of 43 kDa presented high degree homology with gp43, the protein of 27 kDa presented a high degree homology with the hypothetical protein 27-kDa antigenic protein from *P. brasiliensis*, and the protein of 81 kDa revealed no significant homology to *P. brasiliensis* known proteins or to other fungal proteins of known function suggesting a new antigenic protein from this fungus. **Conclusion:** The methodology adopted in this work was able to purify and identify proteins from the protective antigenic fraction (F0) of *P. brasiliensis.*. **Financial support:** CNPq e FAPEMIG

07.017 - STEROL-HYDRAZONE DERIVATIVES AS INHIBITORS OF D^{24}-STEROL METHYL TRANSFERASE

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Introduction: Sterols from fungi, protozoa and higher plants differ from the vertebrate sterols by the presence of an extra alkyl group at C-24. Alkylation of the sterol's side chain is catalyzed by S-adenosyl-L-methionine (AdoMet): $D^{(24)}$-sterol methyl transferase (SMT), a step of interest for the development of specific antifungal agent (Antimicrob. Agents Chemother. 47: 2966-2970, 2003). In this work, we present the syntheses of four sterols (H1, H2, H3 and H4) with more than one nitrogen atom in the side chain (sterol hydrazone derivative), and their effects on growth and sterol profile in the pathogenic yeastlike (Y) phase of *Paracoccidioides brasiliensis*. Structure-activity relationships are derived for these compounds. **Methods:** Sterol-hydrazone derivatives synthesized in the laboratory were: 20-hydrazone-imidazol-2-yl-5a-pregnan-3ß-ol (**H1**), 20-hydrazone-pyridin-2-yl-5a-pregnan-3ß-ol (**H2**), 22-hydrazone-imidazol-2-yl-5-colen-3ß-ol (**H3**), and 22-hydrazone-pyridin-2-yl-5-colen-3ß-ol (**H4**). *P. brasiliensis* was grown in PYG medium at 37ºC for up to 4 days with variable concentrations of each hydrazone, from 0 to 10 mM. For lipid analyses**,** *P. brasiliensis* was cultured either in the absence or presence of each hydrazone at their IC_{50} concentrations. Total lipids were extracted with chloroform/methanol (2:1; v/v). Polar and neutral lipids were fractionated by silicic acid column chromatography. For quantitative analysis and structural assignment, neutral lipids were separated by gas chromatography-mass spectroscopy. The molecular electrostatic potentials MEP were calculated with Spartan 04 employing Density Functional Theory and a 6-31G* basis set. **Results and discussion:** *P. brasiliensis* (Y phase) was sensitive to the action of sterol hydrazone derivatives in the following sequence: H4 (IC_{50}: 0.075 mM)>H3 (IC_{50}: 0.1 mM)>H2 (IC_{50}: 1 mM) =H1 (IC_{50}: 1 mM). On exposure to sterol hydrazones, ergosterol decreased from 76% to 20-30%, while

lanosterol accumulated from 19% to 50%, and 24-methyl sterols depleted, providing evidence that the enzyme $D^{24(25)}$SMT was selectively inhibited. Therefore, the presence of an alkyl substituent in the 24 position of the sterol molecule is an essential feature to fulfil the physiological functions of these molecules in *P. brasiliensis*, as shown before (Antimicrob. Agents Chemother. 47: 2966-2970, 2003). Structure-activity analyses suggest that H3 and H4 are more potent because the extra carbon atom in the side chain confers more flexibility to the hydrazone derivative in the active site of the enzyme SMT. The MEPs at the electronic isodensity 0.002 e/au^3 of H1 varied between -56.84 and 49.39, H2 from -42.82 and 49.46, H3 from -56.82 to 49.55 and H4 from -61.13 and 47.23kCal/mol. Relationship of these values of MEP at this isosurface with the charge distribution of the receptor site will be also presented.

07.018 - IDENTIFICATION OF A METALLOPEPTIDASE ACTIVITY IN *PARACOCCIDIOIDES BRASILIENSIS*

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Introduction and Objectives: *P. brasiliensis* (Pb) is a systemic mycosis, causing severe acute and chronic disease in young adults. Paracoccidioidomycosis (PCM) in Brazil is endemic, mainly affecting rural workers, and is distributed throughout tropical and subtropical regions in Latin America. Invasion of host tissues by Pb involves interactions with the extracellular matrix (ECM). Enzymes, amongst them the metallopeptidases, may facilitate the process of invasion cleaving components of ECM. Thus, identification of metallopeptidases and their activity in PCM may help to understand the pathology of this mycosis and eventually introduce new antifungical drugs against the mycosis. Presently, we identified a metallopeptidase activity in *Paracoccidioides brasiliensis*, which is differently expressed in fungal strains of different virulence. **Methods and Results**: Cell lysates were obtained from two strains, Pb18 (subtypes V - virulent and L - low virulence) and Pb1914, both cultivated in YPD, by disruption with glass beads. Analysis of the enzymatic activity was carried out by quantification of hydrolysis of an internally quenched fluorogenic peptide (Abz-GFSPFRQ-EDDnp) (approx. 20 mM) at 37°C in 50 mM Tris-HCl buffer, pH 7.4, measuring the fluorescence at lem. = 420 nm and lex. = 320 nm in a Hitachi F-2000 spectrofluorometer. Preliminary results showed the activity of a metallopeptidase in all strains tested, totally inhibited by 2 mM orthophenantrolin. Most importantly, the specific activity was directly correlated with strain virulence. The specific activities were 5850 mM/min/mg protein and 206 mM/min/mg protein for Pb18 V and Pb18 L respectively. **Conclusion:** *Paracoccidioides brasiliensis* cell lysates contain a metallopeptidase activity which is higher in more virulent strains. Its relation with Zn-dependent endo-oligopeptidases is being investigated. **Financial support:** CAPES

07.019 - THE EFFECT OF THE ANTIFUNGAL PEPTIDE CHROMOFUNGIN IN *PARACOCCIDIOIDES BRASILIENSIS.*

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Introduction, Objectives, and Methods: *Paracoccidioides brasiliensis* (Pb) is a dimorphic and thermo-regulated fungus which is the agent of paracoccidioidomicosis (PCM), the most important mycosis in Latin America. The pathogenicity of the Pb is consequence of the cellular differentiation process from mycelium to yeast cells during human infection. Several evidences suggest the participation of Ca+2/Calmodulin pathway in this differentiation process. Therefore, the inhibition of calmodulin protein probably affects *P. brasiliensis* survival. Based on these features and considering the importance in characterizing new drugs for the PCM, the aim of this work is to study the molecular interaction of the calmodulin from *P. brasiliensis* (CaM*Pb*) with the chromofungin peptide, natural fragment of chromogranin-A, using the program GRAMM; the *in vitro* effect of this peptide in *P. brasiliensis* using the protocols NCCLS- M27-A2 with adaptations, and the differentiation expression of calmodulin in mycelium and yeast, using northern blot methods. **Results**: The CaM-chromofungin complexes showed that chromofungin tends to bind in the N-terminal domain of CaM*Pb*, but this tendency was not observed for humans' calmodulin. The minimized model of CamPb-chromofugin complex presented conformational changes related with stereochemical and energetic stabilization by forming most orientated electrostatic and hydrophobic interactions in the presence of water molecules. The *in vitro* assays of the action of the chromofungin in *P. brasiliensis* were performed following NCCLS- M27-A2 protocols. The results showed that the chromofungin is able to inhibit the growth of isolated Pb18 (MIC100% = 250 mM), and Pb01 (MIC50% = 7,8125 mM). Conclusion: The results obtained for expression of mRNA of calmodulin in mycelium and yeast indicates that calmodulin is an important protein for dimorphic transition. In addition, these results showed that the calmodulin is more expressed in yeast than in mycelium form. In conclusion, all these results suggest that chromofungin peptide is an efficient drug against *P.brasiliensis*. **Financial support:** MCT, CNPq, FAP-DF, Capes, CNPq, and FUB.