Round Tables

MR1 - CLINICAL IMMUNOLOGY

MODULATION OF CELL ACTIVATION MARKERS AND CYTOKINES IN PATIENTS WITH PARACOCCIDIOIDOMYCOSIS

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Paracoccidioides brasiliensis, a thermo-dimorphic fungus, is the etiologic agent of paracoccidioidomycosis (PCM). The recidive is the greatest obstacle of this disease, because the yeast usually returns after the long treatment period. In the present work, we have investigated the cellular immune response of cells from peripheral blood drawn from patients with different duration of PCM. The classification of patients ranged from no treated to those with long-standing disease over 5 years. No stimulated as well as cells stimulated with phytohemaglutinin or two different antigen preparations, secreted (MEXO) or somatic (PbAg) of *P. brasiliensis*, were characterized. We found that cells from patients with disease proliferate considerably upon stimulation with the antigen preparations and that cells from patients with disease of long duration does not proliferate that vigorously as from patients with more recent diagnosis. Both interferon (IFN)-γ and interleukin (IL)-4 appear to be increased in patients, but IFN-γ tended to increase upon treatment while IL-4-secretion decreased. With respect to CD28 and CD86, we found that the subset of CD28 positive CD8 cells is decreased in all stages of the disease as compared to control individuals. A subset of CD86 positive CD19 cells appeared to be considerably increased compared to the controls. Indeed, our results demonstrated that the treatment of PCM patients promoted a regulation of IFN-γ, IL-4 levels and CD28, CD86 expression bringing new insight to the cellular immune response in PCM.

MODULATION OF THE ANTIGEN-SPECIFIC RESPONSES IN PARACOCCIDIOIDOMYCOSIS PATIENTS: THE ROLE OF CYTOKINES AND COSTIMULATORY MOLECULES

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In order to balance the host-parasite interaction in its favor, *P. brasiliensis* must take advantage of a number of mechanisms to dampen hosts´ immune responses at several checkpoints, either to allow its dissemination from the initial site of infection or to allow its safe persistence within the host for years or decades. In our pursue to dissect such mechanisms we have shown, on one hand, that stimulation with the main *P. brasiliensis* antigen (gp43) of PBMC from patients with active PCM lead to decreased IL-12, IL-2 and IFN-g secretion but high IL-10 levels. In parallel, gp43 induced low (or not at all) IL-12R β2 expression in patients' T-cells, but high expression in controls; *C. albicans* antigen (CMA) did it quite comparably in both groups.

These findings suggest a specific imbalance in the IL-12/INF-γ/IL-10 regulation. In this regard, it has recently been shown that cytokines modulate the expression and activity of the family of "signal transducers and activators of transcription" (STATs): e.g., IFN-γ enhances its functionality. We will show that this also holds true in PCM patients. We will show that there is a severe impairment of STAT1 expression in T cells from patients as compared to controls. This likely may impact the anti-*P. brasiliensis* macrophage activities and the host´s ability in mounting Th-1 type responses.

On the other hand, we have shown that T-cells from patients not only do not proliferate, but undergo apoptosis, upon gp43 stimulation, likely representing a further mechanism by which the yeast evade host´s immunity. To dissect this mechanism, we are now addressing the costimulatory molecules expression in cells from patients and controls. Data obtained up to now show that patients´ CD14+ cells expectedly expressed less CD86 than controls, but unexpectedly, also less CD80, which is known to preferentially engage with CTLA4 and drive negative signaling. These findings were consistently seen in unchallenged or gp43 and CMA challenged CD14+ cells. Analysis of or their counterparts in patients T-cells revealed that in fact patients´ T-cells expressed more CTLA4 after gp43 or CMA stimulation than controls; with unstimulated cells, CTLA4 expression was comparable. In patients´ but not controls´ cells CMA and specially gp43 down-modulated CTLA4 expression. Consistent with this, there was a slight trend for lower CD28 expression in antigen-stimulated patients´ cells as compared to controls. In unstimulated cells the levels of expression were similar. These data suggest that other molecules than CD80 may be involved in the down regulation of the patients´ immune responses and that T-cells from patients and controls react differently regarding CD28 and CTLA4 expression to antigen stimulation. Further experiments to evaluate the link between this abnormal costimulatory pattern and the increased apoptosis of patients´T-cells are warranted.

Financial support: FAPESP, CNPq and LIM-56

PROLIFERATIVE RESPONSE OF LYMPHOCYTES, LEVELS OF CYTOKINES WITH 41 PEPTIDES OF GP 43 AND FREQUENCY OF HLA CLASS II IN PATIENTS WITH DIFFERENT CLINICAL FORMS OF PARACOCCIDIOIDOMYCOSIS

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Synthetic peptides from Gp 43, an immunodominant antigen of *Paracoccidioides brasiliensis*, were tested in proliferation assay of mononuclear cells from 44 patients treated for paracoccidioidomycosis (PCM), and 19 healthy individuals. In parallel, levels of cytokines and HLA class II frequency were analyzed. To determine threshold reactivity of 41 Gp 43 peptides the ROC curve (Receiver Operating Characteristic) was applied, considering a specificity of ≥ 90%. Ten peptides were recognized by 77,3% cells patients and by 26,3% cells of healthy individuals. High levels of IL-10 were detected in supernatants of cells cultures (48 hours) from patients with the multifocal chronic form when stimulated with a pool of peptides (P1 a P5 at 1 mM concentration). Association between HLA class II and paracoccidioidomycosis was analyzed, suggesting that HLA markers may influence host-parasite interaction. Finally, the inclusion of new immunogenic Gp 43 peptides was proposed for therapeutical approach associated with antifungal drugs as new alternatives for the treatment of disseminated forms, particularly in immunosupressed patients.

Key-words: Paracoccidioidomycosis, Synthetic peptides, Glycoprotein 43, Cytokine and HLA (Human Leukocyte Antigen).

KINETICS OF CYTOKINES AND CHEMOKINES GENE EXPRESSION DISTINGUISHES *PARACOCCIDIOIDES BRASILIENSIS* INFECTION FROM DISEASE

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Differences among individuals concerning the patterns of immune responses to *Paracoccidioides brasiliensis* (Pb) are important determinants of disease progression and clinical outcome. Paracoccidioidomycosis (PCM) presents a broad spectrum of clinical and pathological manifestations ranging from benign and localized forms (adult form, AF) to severely disseminated disease (juvenile form, JF). PCM-infection (PI) is defined as an asymptomatic infection caused by Pb in healthy individuals who live in endemic areas and are positive to the paracoccidioidin skin test. We have recently demonstrate that PBMC from JF patients stimulated with Pb antigen produce higher levels of Th2 cytokines IL-4, IL-5, IL-10, and TGF-β1 and impaired secretion of IFN-γ, in comparison to AF patients and PI subjects, in addition to elevated titers of IgG4, IgE and IgA against *P. brasiliensis* gp43 and eosinophilia. In contrast, high levels of IgG1, lower levels of the other Ig isotypes, lower number of eosinophils and a mixed pattern of Th1 and Th2 cytokines characterize AF patients. The pattern of cytokines produced by the PI group is typically Th1: undetectable levels of IL-4, IL-5 and IL-10, and IFN- γ production higher than that of PCM patients. In this study we compared JF (n=10) and AF (n=15) patients with PI individuals (n=15) in relation to the kinetics of mRNA expression of Th1 and Th2 cytokines, IFN-γ-induced chemokines CXCL9 (Mig) and CXCL10 (IP-10) and determined the cells responsible for their production. The time kinetics (0, 3, 6, 12, 24 and 48 hours after PHA stimulus) of IFN-γ, TNF-α, IL-4, IL-10, CXCL9 and CXCL10 mRNA expression was analyzed by a semi-quantitative RT-PCR (normalized by b-actin mRNA). Protein expression was assessed by flow-cytometry. PI individuals expressed earlier and higher levels of IFN-γ, TNF-α, CXCL9 and CXCL10 mRNA when compared to JF patients. In relation to AF patients, the PI group presented similar levels of CXCL10 and IFN-g and higher levels of CXCL9. On the other hand, Th2 cytokines (IL-4, IL-10, IL-5 and TGF-β1) mRNA expression was higher and earlier in JF and AF patients, when compared to PI individuals. At some time intervals it was possible to differentiate JF from AF, mainly in relation to IL-4 and TGF-β1 mRNA, expressed in higher levels in the JF patients. Flow-cytometry analysis confirmed mRNA findings and showed that PI individuals are able to produce high amounts of Th1 cytokines, as IFN-γ and TNF-α following stimulation. Moreover, the analysis of the expression of surface markers showed that the cells expressing IFN-γ were mainly $CD3^+CD8^+$. The importance the CD8 subset in response to Pb infection was recently showed in the experimental model, in which the depletion of $CD8^+$ T lymphocytes resulted in enhancement of the severity and dissemination of the disease, in both susceptible and resistant mice. In addition, patients with pulmonary PCM showed high number of $CD8^+$ T cells in bronchoalveolar lavage, which correlated positively

with the production of CCL3, a chemokine known to promote chemotaxis of lymphocytes, selectively recruiting $CD8^+$ T cells. The higher expression of CXCL10 and CXCL9 confirmed the production of IFN-g by cells in PI individuals. CXCL10 and CXCL9 are IFN-γ inducible and very effectively attract activated T lymphocytes. Both chemokines signal through a common receptor, CXCR3, expressed by memory ($CD45RO^+$) T cells, preferentially of the Th1 subset, and by NK cells. In accordance CXCR3 was expressed in higher levels in T cells from PI group than JF or AF patients. Finally, we found a higher number of IL-10 positive monocytes ($CD68^+$ cells) in both, AF and JF patients, whereas only a basal number of cells were detected in PI individuals. A clear association was observed between the resistant phenotype of PI individuals and the Th1 pole of the immune response characterized by a rapid and strong Th1 response, with an elevated number of cells expressing IFN-γ, TNF-α, CXCL9/CXCL10/CCR3 and low number of IL-10 positive cells. The results also point out to a role for IL-10 in promoting disease progression, since it is elevated in JF and AF patients and can render macrophages refractory to the activating effects of IFN-γ.

Supported by FAPESP

MR2 - EXPERIMENTAL MODELS OF PCM

THE MECHANISMS THAT CONTROL THE IMMUNE RESPONSE AGAINST *PARACOCCIDIOIDES BRASILIENSIS*: THE ROLE OF REGULATORY T CELLS

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The long-term persistence of pathogens in a host is a hallmark of certain infectious diseases, including tuberculosis, leismaniasis, several fungal infectious such as paracoccidioidomycosis (PCM), that is caused by the dimorphic fungal *Paracoccidioides brasiliensis*. Patients with PCM usually show cellular immune hyporesponsiveness to several antigens, including fungal antigens. The mechanisms involved in the immunossupression include low IL-2 production, imbalance on cytokine production, apoptosis through FAS-FAS-L and CTLA-4 engagement. Since CTLA-4 acts as a negative regulator of T cell activation in patients with PCM, and that it is expressed in $CD4^+CD25^+$ regulatory T cells (Treg), here we asked about the possible involvement of Treg in the regulation of the lesions caused by *P. brasiliensis* human patients. We found that 14.8±5.9% of leukocytes found in the skin and mucosal lesions were $CD3^+CD4^+$ cells. From these, 6.1±1.1% co-expressed CD25. Further, $CD4^+CD25^+$T cells also expressed CTLA-4, GITR, CD103, CD45RO, as well as the chemokines receptors CCR5 and CCR4 that is associated with the migration of these cells to infected tissues. Indeed, the expression of TARC was detected in lesions of these patients. Further, the population of $CD4^+CD25^+$ T cells in PBMC from patients with PCM was functional and phenotypic characterized and showed a more exuberant regulatory potential when compared with controls individuals. The data demonstrated that PBMC and lesion-derived $CD4^+CD25^+$ T cells retained suppressive activity *in vitro*. Altogether, these results suggest that regulatory T cells plays a role controlling local and systemic immune response in patients infected with *P. brasiliensis*.

CHARACTERIZATION OF THE PARACOCCIDIOIDAL GRANULOMA EMPLOYING THE EXPERIMENTAL MURINE MODEL

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The present study evaluates by immunohistochemistry the participation of some extracellular matrix (ECM) components and cytokines in the development of granulomatous lesions in resistant (A/J) and susceptible (B10.A) mice ip infected with *P. brasiliensis* (Pb) yeast cells. Marked deposition of fibrous type I and reticular type III collagens forming cocoon-like structures was found circumscribing cellular aggregrates aroung Pb in several granulomatous foci since the earlier stages of the infection. This pattern of expression tended to be enhanced in fibrotic and necrotic lesions at the later phases and to be arranged in thick concentric fibers in compact granulomas, mainly in A/J mice. Semiquantitative data showed higher expression of type I and especially of type III collagens in A/J than in B10.A mice throughout the infection. Decorin was found mainly around macrophages (mf) and multinucleated giant cells (MGC) that were present in the granulomas, surrounding Pb, in A/J and mainly in B10.A mice since the early stages of infection. In A/J mice, positivity was found around fibrotic and necrotic areas of encapsulated and residual lesions containing lysed Pb and in B10.A was less expressed, correlating with the increase in the size and number of lesions during the progression of the disease. Type IV collagen and laminin were found mainly as small diffuse deposits localized around host cells circumscribing Pb in the lesions and were detected on basement membrane of new blood vessels within the granulomas, showing intense vascularization during the infection. Biglycan positivity increased throughout the infection and was observed mainly in MGC and mf containing fungi in granulomas; higher levels were observed in A/J than in B10.A mice. OPN was also strongly (+) in mf and MGC localized mainly in the center of the lesions, with significantly higher degree of (+) cells in B10.A than in A/J at an early phase with significant increase in A/J during the infection. Intense OPN (+) ECM was detected in granulomas of both mouse strains and around necrotic areas in A/J, with significantly higher expression in both strains later than earlier during the infection. Expression of TGF-B and TNF-A was diffuse in the tissue and mainly in mf and MGC, but absent in neutrophils (PMN) and in fibrotic areas in both mouse strains. TNF-A was also found in necrotic areas and in plasmocytes. Expression of TGF-b and TNF-a showed little differences between B10.A and A/J mice by semiquantitative analysis in (+) areas of omentum. Positivity to IFN-γ was detected mainly in lymphocytes at the periphery of lesions. Little or no staining was detected in the necrotic center of the granulomas, on the ECM or in other cell populations. At the beginning of the infection the number of (+) cells was similar in B10.A and in A/J mice but at later times of infection the number of (+) cells doubled in the former but increased 5 times in the later ones. We also studied whether B10.A mice infected with Pb and treated with antifungal drugs had the aspect of the lesions altered to the one observed in A/J mice. Trimethoprim-sulfamethoxazole-treated mice had no granulomas within the splenic parenchyma and only few capsular lesions, mainly composed of pseudoxantomatous macrophages, a cell-type associated with infection under control, whereas untreated mice showed multiple granulomatous foci in the parenchyma, with MGC, plasmocytes and many Pb with well-preserved morphology and abundant budding. Amphoterycin-B treated mice showed lesions on splenic capsule; presence of Pb, necrotic areas and cellular infiltrate containing mainly PMN and mf, whereas no lesions were formed in untreated mice. Also, less severe omentum lesions were detected, with MGC and necrosis, evolving to compact necrotic lesions with numerous degenerated forms of Pb and extensive areas of extracellular material. In contrast, untreated mice presented Pb with preserved morphology and abundant budding contained in multiple lesion foci, with extended necrotic areas, presence of MGC and PMN that increased at the later phase, with deposits of extracellular material and presence of plasmocytes. These overall findings allowed to correlate the expression of ECM components and cytokines in granulomatous lesions, the differential distribution of distinct cell populations, as well as the structure of lesions in less or well organized granulomas, necrosis and fibrosis status, associated with findings of Pb with preserved or altered morphology, indicating progression or resolution of the disease.

COMPARATIVE ANALYSIS OF ISOLATES FROM DIFFERENT GENETIC BACKGROUNDS OF *PARACOCCIDIOIDES BRASILIENSIS*

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Introduction and Objectives: Paracoccidioides brasiliensis is the dimorphic fungus responsible for paracoccidioidomycosis (PCM) in man. The glycoprotein gp43 is the fungal main diagnostic antigen. It also elicits protective cell immunity in immunized mice and is a putative virulence factor due to its adhesive properties. Our lab has previously reported on the existence of an important polymorphism in exon 2 of the *PbGP43* gene, which defined 6 genotypes (A to F). Among 17 fungal isolates originally tested, Pb2, Pb3 and Pb4 contain the most substituted sequences (genotype A). They translate basic gp43, and are phylogenetically distant from the others, whose *PbGP43* sequences show up to 4 substitutions. We presently tested virulence of Pb2, Pb3 and Pb4 in comparison with others bearing genotypes D, E and F, including Pb18.

Methods and Results: We used the susceptible B10.A mouse model preliminarily inoculated by the intraperitoneal (i.p.) route with in vitro-adapted cultures. The following infections were carried out by the intratraqueal (i.t) and intravenous (i.v.) routes with organ-

recovered and in vivo-adapted yeasts. Pb2, Pb3 and Pb4 evoked few deaths i.v. and were recovered from the lungs at colony forming units counts (CFU) lower than 115 at 30 and 60 days of i.t. infection. In vivo-adapted Pb3 proliferated more intensely during similar periods (~3,000 CFU), however the CFU decreased by day 120. The anti-gp43 responses elicited by Pb2, Pb3 and Pb4 were richer in IgG2a, IgG2b and IgG3, suggesting a Th1 predominant type of host immunity. Accordingly, mice infected with Pb3 secreted increasing amounts of IFN-γ in the lungs (up to 120 pg/ml), in contrast with IL-10. The other *P. brasiliensis* isolates tested evoked a progressive type of disease, characterized by increasing CFU recovered form the lungs and a pattern of Th2-driven anti-gp43 immunoglobulins (IgG1 and IgA). Pb5 showed preferences for the spleens and peritoneum, eliciting intense infection when inoculated by the i.p. route and dissemination to these organs during i.t. infection. Pb12 was the most aggressive isolate when inoculated i.t. and i.v. In these animals, pulmonary IFN-γ was undetectable, in contrast with the levels of IL-10 and TGF-β that reached 500 pg/ml. The anti-gp43 response was poor in Pb12-infected animals. This was probably due to down-regulation of the *PbGP43* gene in vivo, as suggested by expression studies.

Conclusions: A broad phylogenetic study (Matute et al., accepted for publication) showed that isolates Pb2, Pb3 and Pb4 compose a minor group of criptic phylogenetic species of *P. brasiliensis*, as suggested previously by the differences in the *PbGP43* sequences. Our results (partially reported in Microbes Infect. 7: 55-65, 2005) suggest that these isolates are also peculiar in terms of host parasite-relationship in the B10.A mouse model, in which they provoked only mild infection when compared with other isolates. We are pursuing the differences in gene and protein expression that could account for such a distinction. The involvement of the gp43 isoforms will also be investigated.

IMMUNE RESPONSE ACTIVATION BY THE ANTI-IDIOTYPIC MAB (AB2 BETA) WHICH MIMICS THE GP-43 FROM *P. BRASILIENSIS*

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Paracoccidioidomycosis is a systemic mycosis endemic in Latin America, with a high prevalence in Brazil, Argentina, Colombia and Venezuela. The etiologic agent of disease is the thermal dimorphic fungus, *Paracoccidioides brasiliensis*. A glycoprotein of 43000 Da (gp43) is the major antigen of *P. brasiliensis*. Antibodies directed to this antigen are detected in the sera of all patients with PCM. Recently, it has been shown that mice immunized with anti-gp43 monoclonal antibodies (MAbs) (Ab1), induce the idiotypic cascade in the gp43 system, which produced both, anti-Id antibodies (Ab2) and anti-anti-Id antibodies (Ab3). To further characterize the idiotypic cascade modulation in mice immunized with anti-gp43 MAb 17c, hybridomas were produced. Ab2 MAbs named 7.B12 inhibited (>95%) the binding of gp43 to MAb 17c (Ab1), suggesting that this anti-Id MAb bind to the idiotope, thus fulfilling the internal image criteria. To elucidate whether Ab2 MAb could act as antigen in serological assays, instead of gp43, sera from PCM patients were tested. Using an ELISA test, it was observed that antibodies from patients and not normal serum bound to Ab2. However, the ELISA test using Ab2 bound to the solid phase made possible to serologically monitor the patients after antifungal therapy, showing an equivalent curve when compared with ELISA test employing purified gp43. Cellular response is more crucial than humoral response in PCM. In this regard, the ability of anti-Id to evoke T-cell mediated anti-*P. brasiliensis* response may be more relevant to effective therapy than its ability to induce a humoral response. Thus, induction of protective cellular response should be included in the criteria to select anti-Id for PCM therapy.

Although the majority of anti-idiotypic antibodies have been used to stimulate antibody responses, they may also stimulate T cells. Unprimed T cells are stimulated by antigen-presenting cells that take up antigen and after processing, present it on their MHC molecules in combination with costimulatory signals. Since the antigen mimicry properties of Ab2β are species-independent, and mouse Ab2β is potentially suitable as a surrogate antigen in human PCM patients, in the present work it was also analyzed whether Ab2β can induce T cell immunity. Our results showed that when mice were immunized whit Ab2β and exposed to gp43 *in vitro*, a specific T cell proliferation was obtained. It was also observed that these cells produced preferentially IFN-γ, a cytokine involved with protection in PCM. Spleen cells from infected mice also proliferate when stimulated in vitro with Ab2β. It is tempting to speculate that Ab2β bearing internal image of gp43 could contain T-cell epitope, which was responsible to induce lymphproliferation. The possibility that some short amino acid sequences could be part of conformational epitope and thus able to induce response against the whole epitope can not be ruled out. These results open several possibilities for applications of Ab2β in serology and therapy. These findings could open new strategies for diagnosis and therapy of fungal infection without the necessity of handling the pathogenic microorganism.

MR3 - CLINICS, DIAGNOSIS AND THERAPEUTICS

OPPORTUNISTIC PARACOCCIDIOIDOMYCOSIS

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Opportunistic infections caused by *Paracoccidioides brasiliensis* are little known, in contrast to the high prevalence of endemic paracoccidioidomycosis in Brazil and in some other Latin American countries. An increasing number of cases have been reported over the last two decades, probably as a consequence of the higher proportion of individuals with depressed cell immunity either due to a disease or to the use of immunosuppressive medications. The association of neoplasia with paracoccidioidomycosis has been observed at a frequency of 0.8% to 14.1% in case series from the Southeast and South of Brazil. The opportunism of *P. brasiliensis* was more easily recognized in 10 patients who had paracoccidioidomycosis one month to seven years after a diagnosis of Hodgkin's disease and of other lymphoproliferative diseases. The clinical signs and symptoms of these patients ranged from localized lesions in the lung and oral mucosa to disseminated infection. Opportunistic infections also occurred in 6 patients who had received a renal transplant, had been medicated with cytotoxic drugs and corticosteroids and had then developed paracoccidioidomycosis-disease 5 to 21 years after immunosuppression. In these cases, the disease is usually limited to the lungs, but the radiologic changes can be atypical, with a predominance of nodular lesions. Standard treatment with antifungal drugs resulted in regression of the paracoccidioidomycosis lesions both in patients with cancer and in patients submitted to immunosuppression after a kidney transplant.

More numerous cases of opportunistic paracoccidioidomycosis were diagnosed in HIV-infected patients and in patients with acquired immunodeficiency syndrome (AIDS) over the last 20 years. Since the first reports in 1989, approximately 120 cases of this co-infection have been observed in Brazil and in other Latin-American countries, especially the Brazilian Southeast. Many patients acquired the infections in the Ribeirão Preto region, Northeast of the State of São Paulo, with an estimated 1.3% prevalence of paracoccidioidomycosis among patients with AIDS. The demographic and clinical data of 53 patients with HIV-1-*P. brasiliensis* co-infection were compared to those of 106 patients not co-infected with HIV-1. Mean age was younger among the co-infected patients (33.5 x 45.2 years), but no significant difference was observed regarding gender (men: 81% x 91%) or ethnic group (whites: 68% x 69%). The co-infected patients resided predominantly in the urban zone of Ribeirão Preto or of nearby municipalities, having less contact with the rural environment than patients with paracoccidioidomycosis not infected with HIV-1 (53% x 77%). Many co-infected patients had a number of circulating T CD_4^+ lymphocytes lower than 100/mm^3. Regarding the clinical signs and symptoms, HIV-1-infected patients usually had more disseminated and rapidly progressing lesions accompanied by fever (81% x 39%). They had more skin lesions (60% x 39%), generalized adenomegaly (42% x 31%), hepatomegaly (55% x 13%), splenomegaly (19% x 4%) and pulmonary infiltrates (74% x 62%). Dysphagia was less frequent in co-infected patients (13% x 36%), as also was hoarseness (2% x 20%) and lesion of the oral mucosa (21% x 51%). The diagnosis of fungal infection was confirmed by histopathological examination (66% of cases) or by the isolation of *P. brasiliensis* (53% of cases) from a skin lesion or from a lesion in other tissues and from secretions of co-infected patients. About 35% of co-infected patients had a negative result when tested for anti-*P. brasiliensis* antibodies by counterimmunoelectrophoresis. Antifungal treatment was performed with sulfamethoxazole-trimethoprim, azole drugs or amphotericin B, with clinical cure being obtained after 12 months in 63% of cases (71% in the non-HIV-infected group). In patients with HIV/AIDS, paracoccidioidomycosis differs from the disease observed in non HIV-infected individuals in terms of epidemiological, clinical and therapeutic-evolutive aspects. The prevalence of co-infection is predicted to increase rapidly over the next few years as the HIV/AIDS epidemics advances towards the rural communities of Brazil and of Latin America. Opportunistic infection with *P. brasiliensis* is an emergent complication that should be recognized as defining AIDS and that should also be part of the differential diagnosis with the infectious diseases that involve immunosuppressed individuals in Brazil ands in other Latin American countries.

DETENTION OF GP43 AND GP70 AS CIRCULATING ANTIGENS OF *P. BRASILIENSIS* AND FOLLOW UP OF PATIENTS UNDER ANTIMYCOTIC THERAPY

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Paracoccidioidomycosis (PCM) is an important systemic fungal disease, particularly among individuals living and working in rural areas of endemicity in Brazil and Latin America, that without antifungal therapy, may develop fatal infection. In some invasive fungal diseases, detection of circulating antigens is a useful approach to the serodiagnosis. Detection of *P. brasiliensis* circulating antigens in body fluids could facilitate the early diagnosis of PCM and confirm a preliminary diagnosis, when antibody detection is inconclusive. Inhibition Enzyme linked immunosorbent Assay (Inh-ELISA) was used to detect gp43 and gp70 antigens, marker molecules of PCM. Monoclonal antibodies produced against these molecules were used in independent analyses, in an attempt of detecting these antigens in different biological samples, such as serum, bronchoalveolar lavage and urine of patients with PCM, as well as in the study of follow-up of patients treated with Itraconazol (ITZ) or Sulfametoxazol+Trimetoprim (SMZ+TMP). Gp43 and gp70 were detected in all the patients (81 samples) at the moment of diagnosis (96.29% and 98.76%, respectively) with mean concentration of 9,87mg/ml and 8,19mg/ml, respectively. In patients with acute and chronic unifocal forms, antigens were detected in 100% of them and in chronic multifocal forms, antigens were detected in 95.31% and 98.43%, respectively. Sera antibody titers from these patients were tested by ID test, and 73 patients (90,12%) were positive. Positive correlation between ID and inh-ELISA was found when antigen concentration and antibody titers were compared. Circulating antigens were detected in other biological samples. In bronchoalveolar lavage samples, circulating antigens (gp43 and gp70) were detected in all samples, (mean concentration of 13,94mg/ml and 7,5mg/m, respectively). In urine samples, circulating antigens (gp43 and gp70) were detected in 87,5% of samples, (mean concentration 8,65 mg/ml and 8,96mg/ml, respectively). Forty and four patients with PCM were followed up during therapy. Twenty and three patients were treated with ITZ (12 months) and 21 with SMZ+TMP (up to 48 months). Circulating antigen levels decreased more quickly in the group treated with ITZ ($p < 0,0001$). At the end of the follow-up, circulating antigens were detected in 5 patients in the group treated with ITZ and in all patients treated with SMZ+TMP. Overall, the sensitivity and specificity of the Inh-ELISA method showed sufficiently high in different biological samples, so that, we concluded that this test can be used for circulating antigen detection in different biological samples for PCM diagnosis and, also, for the follow up of patients under antimycotic therapy.

ANTIFUNGAL DRUGS AND THERAPEUTIC SCHEDULES – IS A CONSENSUS POSSIBLE?

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Paracoccidioidomycosis (PCM) therapeutic approach comprises antifungal drugs, nutritional improvement, immunostimulants, sequelae treatment, and opportunistic infection prevention.

Cotrimoxazole [CMX] (960 or 1,240 mg IV or PO every 12 hrs), amphotericin B deoxycholate [AMBd] (total dose up to 30 mg/kg), ketoconazole [KTZ] (200 or 400 mg PO every day), and itraconazole [ITZ] (100 or 200 mg PO every day) are the recommended antifungal drugs. Indications, contra-indications, efficacy, effectiviness, and side effects are well known. CMX and ITZ are the main drugs of choice and AMBd is indicated only in life threatening cases. However, controlled trials, appropriately randomized, described as double blind with suitable methodology, and with a description of withdrawals and dropouts have been rarely carried out. In the first phase, called initial treatment, patients should be submitted monthly to clinical, hematological, biochemical, serological and radiological evaluation. This phase finishes when clinical cure and normal erythrocyte sedimentation rate are observed. In the second phase, called supplementary treatment, patients should be clinically, serologically and radiologically evaluated every three months. However, there is no consensus about the length of the treatment and the criteria used to determine it. Preliminary evaluation by experts suggests that patients with moderate acute/subacute form and with mild and moderate chronic forms be treated for 2 years, if serology by immunodiffusion test (IDD) becomes negative or decreases up to 1:2 dilution. Severe cases of both acute/subacute and chronic forms, and special cases, such as pregnant patients, should receive an individually designed regimen. These patients should be treated until a negative counter-immunoelectrophoresis (CIE) is reached or one year after a negative IDD. There is a direct correlation among negative CIE and a decrease of IL-10 and an increase of IL-2 and IFN-γ serum levels. An oral antifungal should be introduced right after AMBd completion since there has not been enough time for a recovery of specific cell mediated immunity.

The only immunostimulant evaluated was b-glucan, with good results. It is indicated in severe cases since TNF-a serum levels were not increased. Nutritional status should be recovered and special care should be given to patients with malabsorption syndrome.

Sequelae, such as Addison's disease, and fibrosis and pulmonary emphysema, with obstructive ventilatory disorders, must be carefully treated and followed up. Bronchial infections, bacterial pneumonias and influenza must be prevented in patients with pulmonary sequelae through vaccination against *S. pneumoniae*, *H. influenzae* and influenza virus.

Relapses are not uncommon, mainly, but not exclusively, in cases of early discontinuation of therapy. If clear clinical manifestations and typical *P. brasiliensis* yeast forms are identified a new course of initial and supplementary treatment should be given. When serological relapse occurs, without clinical manifestations or radiologic lung lesions suggestive of active disease, only the supplementary treatment and control should be provided.

A consensus to treat PCM is presently being studied and may be eventually reached.

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DIAGNOSIS OF NEUROPARACOCCIDIOIDOMYCOSIS BY DETECTION OF CIRCULATING ANTIGEN AND ANTIBODY IN CEREBROSPINAL FLUID

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Introduction and objectives: The involvement of the central nervous system (CNS) in PCM, neuroparacoccidioidomycosis (neuroPCM), is secondary to the hematogenous dissemination of the fungus and is more frequent than has been reported . Considering the morbidity associated with the invasive neurological procedures, clinicians are not prone to indicate aspiration or biopsy of CNS lesions. The detection of *P. brasiliensis* antigens in body fluids might facilitate the early diagnosis of PCM, including patients with cerebral lesions.In the present study, gp43 and gp70 antigens of *P. brasiliensis* were detected in cerebrospinal fluid (CSF) and sera samples from patients with NeuroPCM by using an inh-ELISA, and the results were compared with those obtained for anti-*P. brasiliensis* antibodies detected by immunodiffusion (ID) and conventional ELISA tests.

Methods and results: Cerebrospinal fluid (CSF) and serum samples were obtained from 14 patients with neuroPCM. Control groups included 10 CSF samples from patients with non-infectious neurologic diseases and 30 serum samples from healthy volunteers.

Exoantigen from *P. brasiliensis* B-339 was produced**.** Gp43 was purified from the Pb B-339 crude exoantigen and gp70 was purified from Pb 113 cytoplasmic antigen. MAb anti-gp43 and Mab anti-gp70 were a gift from R. Puccia and JD Lopes**.** Inh-ELISA was performed

to detect antigens. ID-tests were performed with CSF and serum samples at the moment of diagnosis of NeuroPCM. ELISA tests were performed to detect antibodies.

Table sumarises the results:

Subjects	CSF antigen Concentration [a] (µg/ml)		CFS (ELISA)[b]		Sera antigen Concentration [a] (µg/ml)		Antibody titer (ID)	
	gp43	gp70	anti-gp43	anti-gp70	gp43	gp70	CSF	Sera
Case 1	19.5	6.75	+ (1:1600)	+ (1:1600)	NA	NA	NR	NA
Case 2	24.0	6.75	+ (1:400)	+ (1:50)	NA	NA	NR	NA
Case 3	0.46	0.43	+ (1:200)	+ (1:50)	NA	NA	NR	NA
Case 4	3.75	1.83	+ (1:50)	+ (1:50)	2.85	1.03	NR	NR
Case 5	21.0	6.75	+ (1:6400)	+ (1:400)	5.27	1.49	NR	1:8
Case 6	18.0	4.16	+ (1:200)	+ (1:50)	5.27	2.49	NR	1:4
Case 7	22.5	3.39	+ (1:50)	+ (1:50)	0	1.03	NR	NA
Case 8	13.5	2.67	+ (1:50)	+ (1:50)	1.21	0	NR	NA
Case 9	16.5	8.25	+ (1:50)	+ (1:50)	4.16	1.77	NR	+NDS
Case 10	19.5	7.5	+ (1:200)	+ (1:50)	1.03	11.25	NR	+NDS
Case 11	30.0	16.5	+ (1:200)	+ (1:50)	4.16	1.68	NR	NR
Case 12	30.0	13.5	+ (1:50)	+ (1:50)	7.12	2.49	NR	NR
Case 13	25.5	5.64	+ (1:50)	+ (1:50)	12.75	8.25	NR	NR
Case 14	25.5	6.38	+ (1:51200)	+ (1:50)	6.75	11.25	1:16	1:16
Mean (µg/ml)	19.3	6.8			4.6	4.0		
Control group A*	0	0	(-)	(-)				
Control group B**					0	0	NR	NR

a, antigen detection by inh-ELISA; **b,** antibody detection by conventional ELISA; **CSF,** cerebrospinal fluid; **ID,** immunodiffusion; **NR,** non reactive; **NA,** not available; **+NDS,** positive with not diluted sera; **(-),** negative.

* = 10 CSF from non-infections neurologic diseases were negative for antigens and antibodies (control patients)

** = 30 serum samples from healthy volunteers were negative for antigens and antibodies (control patients)

Conclusion: Our results suggest that monitoring specific antigens of *P. brasiliensis* may be helpful to define the diagnosis of NeuroPCM. It was found that both gp43 and gp70 were detectable in almost all the serum samples with small differences in their mean antigen concentrations (4.6 and 4.0 mg/ml, respectively). On the other hand, antibody detection was negative when tested by ID but was positive against both antigens when tested by ELISA, despite at low titers. This data, from the limited number of patients studied, allow us to assume that the detection of antigen by inh-ELISA or the detection of specific antibody by conventional ELISA may be equally sensitive to identify NeuroPCM. Since antigen values for gp43 were always higher than those found for gp70, assaying only for gp43 may prove sufficient for that purpose.

Detection of antibody by conventional ELISA, as a routine laboratory work, is a less cumbersome and time-consuming procedure than the detection of antigen. Hence, testing CSF samples for antigens would be recommended only when a suspected patient presents either negative or inconsistent results for antibody detection.

With the scarce clinical and radiological information available about the enrolled patients, no correlation could be ascertained between antigen levels and severity of the disease. Also, further studies are necessary to validate the potencial usefulness of antigen detection for monitoring the patients' response to antifungal therapy.

Acknowledgement: This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

MR4 - OTHER ENDEMIC MYCOSES

EMERGING COCCIDIOIDOMYCOSIS IN NORTHEAST BRAZIL

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Coccidioidomycosis is a systemic mycosis caused by the dimorphic fungus *Coccidioides immitis*, affecting humans and a wide variety of animals. It is endemic in many countries of the American continent, characteristically restricted to semiarid or desert-like regions ranging from 40° N to 40° S. The highest prevalence of the infection occurs in the Southwestern states of USA and the Northern Mexican states. Endemic foci are also known in Central and South America. Although the first two Brazilian human cases were reported in 1978 and 1979, only in 1998 Brazil was included in the map of geographical distribution of coccidioidomycosis [5], after the first reports of small outbreaks that occurred in the states of Piauí and Ceará [6,8,9].

In 1999 Wanke et al. recorded a total of 14 cases coming from 4 different Brazilian states [8]. Since then, several more cases were diagnosed, but only three of them, fatal cases, have been published [1,3,7]. Until December 2004 a total of 79 cases have been diagnosed in patients coming from the states of Piauí (66), Ceará (6), Maranhão (5) and Bahia (2). More recently Moreira et al. (2005) reported three additional cases from Ceará, including a fatal case [4]. While the first Brazilian cases had the diagnosis based only on histopathologic findings, the more recent cases have been diagnosed on the basis of mycological exams (direct microscopy and culture), serology (immunodiffusion tests) and histopathology. The predominance of cases diagnosed in the state of Piauí probably reflects a collaborative multidisciplinary study group integrating doctors and technicians of the Federal University in Piauí (UFPI) and the Oswaldo Cruz Foundation (FIOCRUZ) in Rio de Janeiro, relying on a good laboratory support for the mycological diagnosis.

The analysis of epidemiological and clinical data of the coccidioidomycosis cases in Brazil reveals that: (i) most cases were young adult male without any evidence of immunodeficiency; (ii) all cases presented with pulmonary involvement and only recently cases with dissemination to CNS, bone, joints and skin were diagnosed; (iii) the overall lethality rate of ≥ 10% (9/82) is very high; (iv) the most prevalent (about 90%) risk activity is hunting armadillos (mainly *Dasypus novemcinctus*) and digging them out of their burrows; (v) almost all cases occurred in events that involve one or more individuals at risk activity in more than 36 different counties of four Brazilian states: Piauí 28, Ceará 6, Maranhão 3 and Bahia 2.

So far, the mycosis has been diagnosed in humans, dogs and armadillos, and *C. immitis* has been isolated from soil samples collected in and around armadillo burrows in the state of Piauí [2,8,9]. However, the semi-arid NE region of Brazil also encompasses large areas of the states of Sergipe, Alagoas, Pernambuco, Paraíba and Rio Grande do Norte with similar climatic conditions as observed in Piauí.

In conclusion, we are convinced that coccidioidomycosis is actually underdiagnosed in Brazil and that the endemic area is expected to be larger than the cases point now.

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EFFICACY OF CELL-FREE ANTIGENS TO EVALUATE CELL IMMUNITY AND TO INDUCE PROTECTION IN A MURINE MODEL OF HISTOPLASMOSIS

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Histoplasma capsulatum is a dimorphic pathogenic fungus that causes a wide spectrum of disease when mycelial fragments are inhaled. Resistance to *H. capsulatum* is dependent on a cellular immunity mediated by T cells and macrophages. Here we standardized the production of extracts containing cell-free antigens (CFAgs) and observed their efficacy in evaluating cell-immunity during murine histoplasmosis. CFAgs induced a more potent DTH response in *H. capsulatum*-infected mice than did histoplasmin - a classical antigen. This DTH response to CFAgs is able to determine the immune status of infected mice and to predict their death. Moreover, CFAgs stimulated in vitro spleen cells from immune mice to produce high amounts of gamma interferon (IFN-γ). Finally, the immunization with CFAgs protected against a lethal inoculum of *H. capsulatum*. These results demonstrate that CFAgs may be useful for the evaluation of cellular immune response and as a potential source for the development of a vaccine against histoplasmosis.

MOLECULAR ASPECTS AND EPIDEMIOLOGY OF *CRYPTOCOCCUS GATTII* IN RIO GRANDE DO SUL

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Cryptococcosis is an opportunistic fungal infection caused by the encapsulated yeast, *Cryptococcus neoformans* and *Cryptococcus gattii*. This disease predominantly affects immunocompromised patients but also occur in healthy individuals as well. *C. neoformans* infection results from inhalation of spores or desiccated yeast cells, which may be accompanied by dissemination and clinical disease that usually manifests itself as meningitides. This pathogen is classified into two species: *C. neoformans* var. *neoformans* (serotypes A, D and AD), with worldwide distribution, and *C. gattii* (serotypes B and C), which until the outbreak on Vancouver Island, was considered to be restricted to tropical and subtropical climates. Serotype A has been proposed to be separated from *C. neoformans* var. *neoformans* into a new distinct variety named *C. neoformans* var. *grubii*. In Brazil, both species of the fungus are recognized as etiological agents of cryptococcosis. According to official data, 4.5% of the cases of AIDS related opportunistic infections are caused by *C. neoformans* in Brazil. The aim of the group is to investigate the population structure of clinical and environmental *C. neoformans* and *C. gattii* isolates from South Brazil to extend the knowledge about their ecology, molecular biology and epidemiology, in this region.

To study the potential saprophytic sources of these yeasts we analyzed 55 fecal samples from 59 species of captive birds kept in cages at the Sapucaia do Sul Zoological Garden. Thirty-eight environmental isolates were obtained from 10 samples (18.2%). Differences in capsule size and colonial morphology were observed. The isolates were typed as *C. neoformans* var. *grubii* serotype A (86.8%), molecular type VNI and, surprisingly, as *C. gattii* serotype B (13.2%), molecular types VGI, suggesting an alternative ecological niche of the *C. gattii*.

To further explore the differences between *C. neoformans* and *C. gattii* a representational difference analysis (RDA) was performed to identify differences between this two genomic DNA populations. Based on successive rounds of subtractive hybridization followed by PCR, RDA enriches for, and permits the isolation of DNA fragments that could be used to differentiate both species. After three rounds of subtractive hybridization and PCR, 96 clones were sequenced and 43 were selected as possibly specific for *C. gattii* were further analyzed by dot-blot and Southern blot.

Apoio financeiro: CAPES, CNPq e FAPERGS.

HISTOPLASMOSIS: UPDATE ON THE LABORATORY DIAGNOSIS

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Endemic mycoses can be challenging to diagnose, and accurate interpretation laboratory data is important to ensure the most appropriate treatment for the patients. Although the definitive diagnosis of histoplasmosis (HP), one of the most frequent endemic mycoses in Brazil, is represented by direct diagnosis performed by micro and/or macroscopic observation of the fungi, serologic evidence of these fungal infections is important since the isolation of the etiologic agents is time-consuming and lacking in sensitivity. A variety of immunoassays have been used to detect specific antibodies. Among them, the most applied technique to antibodies detection is the immunodifusion with a range of sensitivity and specificity varying between 70 to 100% and 70 to 100% respectively, depending on the clinical form. The complement fixation (CF) test, a methodology extensively used on the past, lacks specificity (70 to 80%). Detecting fungal antigens by immunoassays also presents a value tool for the diagnosis of the endemic mycoses associated to patients with depletion of their immune system with up to 95% of specificity. Most current tests in diagnostic laboratories still utilize unpurified antigenic complexes from either whole fungal cells or their culture filtrates. Emphasis has shifted, however, to clinical immunoassays using highly purified and well-characterized antigens including recombinant antigens. We shall review the current conventional tools, such as complement fixation and immunodiffusion for measuring immune responses in the histoplasmosis, and outline the development of novel diagnostic reagents and methods, as well discuss their relative merits and disadvantages to the immunodiagnostic of this mycosis.

MR5 - MOLECULAR BIOLOGY

ELUCIDATION OF THE CAMP-SIGNALLING PATHWAY THAT CONTROLS MORPHOLOGICAL SWITCHING IN *PARACOCCIDIOIDES BRASILIENSIS*.

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Paracoccidioides brasiliensis is a dimorphic human pathogenic fungus that undergoes a complex transformation *in vivo*, with mycelia in the environment producing conidia, which act as infectious propagules upon inhalation into the lungs, where they transform to the pathogenic yeast form. In several fungi the cAMP signalling pathway has been shown to be important in controlling morphological changes and pathogenicity. In *P. brasiliensis* we have established that exogenous cAMP retards the mycelium to yeast transition, whilst previous studies have revealed that strains unable to undergo the this morphological transition are avirulent. With a view to investigating the role of the cAMP signalling pathway in controlling morphological transitions in *P. brasiliensis* we have cloned several components of this pathway; including the *cyr1* gene that encodes adenylate cyclase (AC); a *ras* gene; three genes, *gpa1-3*, that encode G_{α}-proteins*; gpb* and *gpg* genes that encode G_{β} and G_{γ}-proteins; and a gene, *tpk1*, that encodes catalytic subunits of protein kinase A. The transcript levels for all of these genes have been determined by real-time RT-PCR. This analysis revealed the differential expression of *cyr1*, *tpk1*, *gpa1*, *gpa2*, *gpa3* and *gpg1*, which were expressed at higher-levels in yeast; with *cyr1* and *tpk1* transcript levels 7.6-fold and 1.7-fold higher, respectively, whilst the transcripts for the G-proteins were all about 4-fold higher. However, monitoring the transcript levels during the morphological transition revealed more subtle behavior. The *gpa1*, *gpa2* and *tpk1* transcripts transiently peaked at about a 2-fold higher level after 10 hours; but there was a more significant transient peak in the *cyr1* and *gpb1* transcripts, which were 4 and 5.5-fold higher, respectively, after 24 hours; and there was a further, but smaller, transient peak in the *gpb1* and *tpk1* transcripts after 120 hours. The timing of these fluctuations in gene transcription can be correlated with the formation of four distinct morphological forms of the fungus that are apparent in the transition and which we term mycelium, differentiating mycelium, transforming yeast and yeast. After inducing the morphological switch by increasing the incubation temperature of mycelium from 26°C to 37°C, the on-set of mycelial differentiation occurs after about 12 hours, with most mycelia undergoing differentiation by 24 hours; whilst transforming yeast appear after 48 hours and peak after 120 hours, leading to a lag in the appearance of yeast, which predominate after 336 hours. This behavior is consistent with a series of signaling events, each activated by a different gene, that trigger subsequent stages of the morphological differentiation process. The behavior of *gpb1* is particularly informative: although it was not differentially expressed in mycelium and yeast, the transcript levels transiently increased with peaks after 24 and 120 hours; thus, indicating an imbalance in the ratio of Gpa1:Gpb1, particularly after 24 hours when *gpb1* expression had increased 5.5-fold but *gpa1* expression had declined by 2-fold from that in mycelium. This would presumably lead to an increase in the level of 'free' Gpb1 during the morphological transition that could act independently or form complexes with Gpa2 and/or Gpa3. To address this question we have used 2-hybrid analyses to establish that Gpb1 only interacts with Gpa1 and that both can interact with the N-terminus of AC. Our hypothesis is that Gpb1 binds to and modulates the activity of AC to govern the morphological switch. Indeed, the addition of exogenous cAMP, which retards the morphological switch, suppresses the *gpa1*/*gpb1* imbalance but stimulates *cyr1* transcription, which peaks after 72 hours instead of 24 hours. In effect, there is a switch from Gpa1 to Gpb1 and back to Gpa1 signalling during the transition.

TRANSCRIPTION PROFILING OF *PARACOCCIDIOIDES BRASILIENSIS* CELLS UNDERGOING THE HYPHAL-TO-YEAST TRANSITION

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Introduction and objective: *Paracoccidioides brasiliensis*, a thermodimorphic fungus, is the causative agent of paracoccidioidomycosis, a prevalent systemic mycosis in Latin America. Pathogenicity appears to be intimately related to the dimorphic transition. Our

main objective is the molecular characterization of genes preferentially expressed during the *P. brasiliensis* mycelia-to-yeast transition.

Methods and Results: We developed and implemented a 4,600 elements cDNA-microarray to identify genes that display differential expression during *P. brasiliensis* hyphal-to-yeast transition triggered by a change in the temperature from 26 to 37 ºC. This microarray was constructed by using as elements representatives from clusters derived from *P. brasiliensis* yeast ESTs. Statistical analysis of the data generated by these hybridization experiments showed 2,583 genes that displayed modulation in at least one experimental time point from 5 to 120 hours after temperature shift. The genes identified are involved in branched and aromatic amino acid catabolism, signal transduction, development, growth and oxidative stress protection. The expression of several of these genes was validated by real-time RT-PCR experiments using RNAs derived from hyphal-to-yeast transition in either complete media or minimal medium. As a preliminary step to understand if aromatic amino acid catabolism is essential for the hyphal-to-yeast transition, we decided to inhibit an enzyme in this pathway (4-hydroxyphenylpyruvate dioxygenase) by adding to growth cultures an analogue, nitisinone. This drug showed to be able to inhibit both yeast growth and hyphal-to-yeast dimorphic transition.

Conclusion: These experimental procedures provided the identification of genes with increased mRNA expression during this transition.

Financial support: FAPESP and CNPq, Brazil.

GENES EXPRESSED IN THE PATHOGENIC PHASE OF *PARACOCCIDIOIDES BRASILIENSIS* AND THEIR POTENTIAL INVOLVEMENT IN VIRULENCE AND MORPHOLOGY

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Paracoccidioides brasiliensis, a thermodimorphic fungus, is the causative agent of paracoccidioidomycosis (PCM), the most prevalent systemic mycosis in Latin America. Like in other pathogenic dimorphic fungi, its pathogenicity appears to be related to the dimorphic transition from the hyphal to the yeast form, which is triggered by a shift from environmental temperature to the temperature of the mammalian host. So far, information on genes necessary for the establishment and morphology of the pathogenic phase in *P. brasiliensis* is not certain, although valuable and increasing information on genes preferentially expressed on this phase is been given by the groups working on the construction of expressed sequence tags (ESTs) libraries on this fungus (Yeast 20:263-271, 2003; Eukariotic Cell 2:34-48, 2003; Mol. Gen. Genomics 271:667-677, 2005; J. Biol. Chem. 280:24706-24714, 2005)

In this work, we present three genes expressed only in the pathogenic phase of *P. brasiliensis*, on which our group has been working in the last few years. Two of them (*PbrAGS1*, for alpha-1,3-glucan synthase and *PbrCHS3* for a class III chitin synthase) are related to the synthesis of cell wall components and are potentially involved in morphology and virulence of the fungus. Preliminary experimental data prompt us to speculate on a possible post-transcriptional regulation for *PbrAGS1*. The deduced product of the third gene, *PbrCBP1*, presents identity with a *Histoplasma capsulatum* calcium binding protein (Mol. Microbiol. 27:531-539, 1998), involved in virulence in the yeast phase of this dimorphic fungus (Science 290:1368-1372, 2000). Also, our group has been working on the testing of several antifungal and potential antifungal drugs, one of them caspofungin (Cancidas, Merck). To our surprise, caspofungin, a beta-1,3-glucan synthase inhibitor, affected not only the mycelial phase as expected, but also the yeast phase of *P. brasiliensis*, despite the low amount of this glucan in the yeast cell wall. Micrographs show damage to the cell wall of the yeast phase. When we explored by northern the expression of one of two reported beta-1,3-glucan synthase genes, *FKS1* (Yeast 16:451-462, 2000), we found to our surprise, that *FKS1* has a higher expression in the yeast phase than in the mycelial phase, which together with the results mentioned above from the caspofungin microbiological experiments, made us wonder if beta-1,3-glucan, even when represents a small percentage of the yeast cell wall components, would be essential for its maintenance.

TRANSCRIPTONAL PROFILES OF THE HUMAN PATHOGENIC FUNGUS *PARACOCCIDIOIDES BRASILIENSIS – PB01* IN MYCELIUM, YEAST AND MACROPHAGE CELLS.

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Paracoccidioides brasiliensis is the causative agent of paracoccidioidomycosis, a disease that affects 10 million individuals in Latin America, with about 2% developing the disease (Restrepo *et al*. *Med. Mycol.* 39, 233-241, 2001). *P. brasiliensis* genome size was estimated to be ~30 Mb. A study of *P. brasiliensis* gene density suggests that this fungus contains about 7,500 to 9,000 genes (Reinoso *et al*., IV *Congresso Virtual de Micologia de Hongos patógenos em América Latina*, 2003), which is in agreement to the estimated gene number for ascomycete fungi genomes. This report depicts the results of the analysis of 6,022 assembled groups (expressed genes) from mycelium and yeast phases ESTs, covering about 80% of the estimated genome of this dimorphic, thermo-regulated fungus [Felipe *et al*., *J. Biol. Chem.* 280(26), 24706-24714, 2005]. Drug targets and genes related to virulence and pathogenicity were identified. The majority of genes involved in signal transduction pathways – cAMP/PKA, Ca^{+2}/calmodulin and MAP-Kinases – possibly participating in cell differentiation and infection were annotated and now we are able to describe the corresponding signaling systems in *P. brasiliensis.* Key differentially expressed genes related to the control of cell organization and ion transport are described as well as their pattern of expression in mycelium and yeast cells as confirmed by northern blot (Vieira *et al*., *in preparation*).

The data provide a comprehensive view of the fungal metabolism including over expressed transcripts, stage-specific genes and also those that are up or down-regulated as assessed by *in silico* electronic subtraction, cDNA microarrays and some were confirmed by northern blot. The analysis compares the two fungal cell-types as well as their metabolic behavior. The transcription profile of the mycelium infective phase suggests the shunting of pyruvate into aerobic metabolism, since the expression of the ESTs encoding enzymes of the TCA cycle are up regulated in this fungal phase. In contrast, the yeast transcription profile evidenciated the deviation of pyruvate from the glycolytic pathway into anaerobic metabolism, this observation is consistent with a lower oxygen level in infected tissues. The results obtained probably reflect the adaptations associated with the mycelium (soil) and yeast (human host) environments.

In addition, experiments were carried out in order to study the *in vivo* expression profile of the differentially expressed genes in macrophages cells infected by *P. brasiliensis*. The fungus act as a facultative intracellular pathogen being able to survive and replicate within the phagosome of nonactivated murine and human macrophages. This ability, as proposed for *Histoplasma capsulatum* and *Mycobacterium tuberculosis*, is crucial to the development of disease. By using cDNA microarray we evaluate the transcriptional response of this fungus to the environment of peritoneal murine macrophages (Tavares *et al*. *in preparation*). *Ex vivo* peritoneal murine macrophages were infected with *P. brasiliensis* yeast, at different time points. Our results show that at 6 hours post infection 40 genes are significantly up regulated and 173 down regulated when comparing intracellular versus *in vitro* grown yeast cells. Among those up regulated genes we were able to find genes that may be directly involved in the ability of *P. brasiliensis* to survive within macrophages such as 60 kDa heat shock protein (Hsp60) and cytosolic Cu/Zn superoxide dismutase (CuSOD). For the first time a global gene expression tool is reported for the expression analysis of *P. brasiliensis* genes when inside host cells. Our data analyses suggest a transcriptional plasticity of *P. brasiliensis* in response to the harsh environment of macrophages which may lead to adaptation and consequent survival of this pathogen.

Support – MCT/CNPq, FAP-DF, CNPq, CAPES, Fapesp, Fundect, FUB, UFG.

MR6 - CELLULAR BIOLOGY OF Paracoccidioides brasiliensis

NEW MOLECULES OF *PARACOCCIDIOIDES BRASILIENSIS*: INVESTIGATION OF THEIR ROLES IN THE FUNGUS-HOST INTERACTION.

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Paracoccidioides brasiliensis is a dimorphic fungus alternating between the mycelia form found at room temperature and the yeast phase detected in infected tissues or in cultures at 37^0 C. The fungus undergoes a complex differentiation in vivo, with conidia acting as infectious propagules upon inhalation into the lungs, where they differentiate into the pathogenic yeast phase. The disease is the result of an intimate relationship between host and pathogen. During the infective process *P. brasiliensis* should be able to sense changes in the environmental parameters in the host milieu, changing the expression of specific genes that would be required for the in vivo survival and growth. Defining genes differentially expressed by human pathogens during the phase of infection should help elucidate those that are integral to pathogenesis and virulence. Indeed the repertoire of genes related to the host interaction must include those that confer the ability to change the cell morphology, to adhere to the host cells and those with proteolytic activity, among others. We have been focusing on proteomics and transcriptome approaches to investigate the *P. brasiliensis* interaction to the host. Immunoproteomic approaches allowed the characterization of molecules such as a 60-kDa-heat shock protein (*Pb*HSP60) and two enzymes of the glycolytic pathway, glyceraldehydes 3-phosphate dehydrogenase (*Pb*GAPDH) and triose phosphate isomerase (*Pb*TPI). All molecules are preferentially expressed in the yeast parasitic phase and the transcripts and protein levels are accumulated during the mycelium to yeast transition. *Pb*GAPDH and *Pb*TPI are located on the cytoplasmic compartment and at the cell wall of the yeast cells, as detected by immunoelectron microscopy. Both proteins can interact with extracelular matrix proteins, such as laminin and fibronectin. The recombinant purified proteins, rGAPDH and rTPI, as well as, the respective polyclonal antibodies inhibited the adhesion and subsequent invasion of *P. brasiliensis* to in vitro cultured cells. Moreover, the immunization with the recombinant *Pb*HSP60 confers protection in an animal model against either non-lethal or lethal intranasal inoculation with *P. brasiliensis* yeast cells. As an additional step in the understanding mechanisms of *P. brasiliensis* interaction to the host we have generated, sequenced and analysed cDNA libraries from conditions predicted to resemble phases of the fungus interactions to the host. cDNA representational difference analysis (cDNA-RDA) between yeast cells grown in vitro for several years and the fungus isolated from mice liver or inoculated in human blood and plasma, was used to characterize the most expressed transcripts during conditions that mimic the human infection. The GAPDH transcript presented a high redundancy in yeast cells recovered from mice liver. In addition, new molecules, potential virulence factors, were characterized whose transcripts were highly abundant in the subtracted cDNA libraries. The involvement of *Pb*GAPDH and *Pb*TPI as adhesins provides insights into the organism mechanisms of adherence and colonization. Those proteins could be crucial for the fungus initial adherence and in the dissemination of the disease. The *Pb*HSP60, a member of the hsp60 family in *P.brasiliensis*, is a protective immunogen from the fungus yeast phase. The combined findings have generated a subset of new data and genes that can now be further investigated and reflect the participation of several proteins in the infective process of *P. brasiliensis*.

Supported by: MCT/CNPq and SECTEC/GO.

PARACOCCIDIOIDES BRASILIENSIS : INTRA OR EXTRACELLULAR FUNGUS?

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There are direct and indirect evidences that *P.brasiliensis* (Pb) is mainly a facultative intracellular parasite, which could also present temporal and spatial phases of extracellular existence as yeast form in the tissues during the infection. Direct evidences are exemplified by microscopic analysis of infected tissues and culture cells through bright field, electron and confocal microscopies. Otherwise, the types of inflammation that occur in *P. brasiliensis* infection attest the state of the fungus, because the local types of tissue inflammation are adapted or adjusted to its extracellular or intracellular existence within host tissues. Extracellular phase evoke primarily an acute inflammatory response in which neutrophils are the predominating cells. Another type of local tissue inflammation is characterized by the host's attempt to sequester and try to destroy a parasite that is also adapted to an intracellular existence. The reaction to this type of existence is somewhat delayed in comparison to the acute inflammatory process, which may at times precede it; and it is characterized by the predominance of lymphocytes, monocytes/macrophages, and fibrocytes, rather than neutrophils. The pathologic lesion which develops, particularly if the phagocyte is unable to destroy all the invaders quickly, is frequently characterized by the formation of granulomas. *P. brasiliensis* is clearly a tumor-like organism with G (growth) - I (invasion) - M (metastasis) phenotype (G+I+M+). Most of this phenotypical characteristic is dependent on intracellular phase in phagocytic cells, including the growth in the tissues, intrapulmonary displacement and intra-arterial and lymphatic invasion. The intrapulmonary displacement of Pb is carried out by macrophages that migrate from alveolar areas close to terminal bronchioles to the perilymphatic and/or peri-arteriolar spaces. The mechanisms of intravascular migration (intravasation) appear to be secondary to extracellular digestion of vascular wall components by fungal proteases. Intravascular lodgment with further extravasation, on another hand, could be dependent on interaction either directly or indirectly (via platelet, white blood cells, or fibrin) with endothelial lining. It is little known the effect of the Pb intracellular existence on cytoskeleton, specific pattern of overall gene and protein expression, and requirement for survival and growth in the parasitized cells. Some of these problems could be solved by genomic and proteomic procedures applied comparatively in infected versus non infected cells isolated by laser capture microdissection.

INTERACTIONS OF EXTRACELLULAR MATRIX PROTEINS WITH *PARACOCCIDIOIDES BRASILIENSIS*

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Microorganisms adhere to extracellular matrix proteins by means of their own surface molecules. *Paracoccidioides brasiliensis* conidia are capable of interacting with extracellular matrix proteins. We searched for the presence of fungal proteins that could interact with extracellular matrix protein and, if found, attempt their purification and characterization.

Previously, Immunofluorescence microscopy studies had demonstrated that soluble fibronectin, fibrinogen and laminin were bound to the surface of conidia and to a lesser extent, also to mycelial fragments. In addition, conidia were also able to bind in a concentration dependent manner. to these proteins immobilized on microtiter plates Polyclonal antibodies directed against each one of the proteins tested inhibited adherence of conidia to bound fibronectin, fibrinogen and laminin, as did the same proteins when added in soluble form, except in the case of fibrinogen, This interaction was inhibited by the presence of the fibrinogen degradation fragment D. Various monosaccharides and the peptides RGD and RGDS had no effect on adherence of conidia to immobilized ECM proteins. However, N-acetylneuraminic acid hindered adherence to all the proteins tested, indicating that the recognition was mediated through a sialic acid dependent process. Pre-treatment of conidia with laminin derived peptides IKVAV and CDPGYIGSR resulted in significant inhibition of the interaction between conidia and laminin.

Various extracts prepared from *P. brasiliensis* mycelial and yeast cultures (total homogenates, β-mercaptoethanol, and sodium dodecyl sulfate [SDS] extracts) were analyzed by ligand affinity assays with fibronectin, fibrinogen and laminin. Two polypeptides were detected in both fungal forms and their corresponding SDS extracts were shown to interacted with all the extracellular matrix protein tested. Their molecular masses were 19 and 32 kDa. Analysis of the N-terminal amino acid sequence of the purified 32-kDa mycelial protein showed substantial homology with *P. brasiliensis*, *Histoplasma capsulatum*, and *Neurospora crassa* hypothetical proteins. Additionally, a monoclonal antibody (MAb) produced against this protein recognized the 32-kDa protein in the SDS extracts of both fungal forms by immunoblot. Immunofluorescence analysis revealed that this MAb reacted not only with mycelia and yeast cells, but also with conidia, indicating that this protein was shared by the three fungal propagules. By immunoelectron microscopy, this protein was detected in both cell walls and cytoplasm. Both the 32-kDa purified protein and the corresponding MAb inhibited the adherence of conidia to the three extracellular matrix proteins in a dose-dependent manner.

These findings demonstrate the interaction between *P. brasiliensis* conidia and ECM proteins appears mediated by both sialic acid dependent and independent mechanisms; also, the presence of two polypeptides capable of interacting with extracellular matrix proteins on the surface of *P. brasiliensis* propagules, indicates the presence of common receptors for laminin, fibronectin, and fibrinogen.

These proteins (laminin, fibronectin and fibrinogen) may be crucial for initial conidial adherence to host tissues and may as well play a role in dissemination of paracoccidioidomycosis.

EFFECTS OF NEW AND AN OLD ADHESINS IN CYTOSKELETON, APOPTOSIS AND SIGNALING. ADHESION AND INVASION OF *PARACOCCIDIOIDES BRASILIENSIS* IN EPITHELIAL CELLS

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Introduction and Objectives: Microbial virulence is generally considered to be multifactorial with infection resulting from the sum of several globally regulated virulence factors. Paracoccidioidomycosis presents a variety of clinical manifestations, and *Paracoccidioides brasiliensis* can reach many tissues, mainly the lungs. The ability of the pathogen to interact with the host superficial structures is essential to further colonization, invasion and growth. Epithelial cells represent the first host barrier or the preferential site of the fungus entrance. For this reason, interactions between *P. brasiliensis* and Vero/A549 epithelial cells were evaluated, with emphasis the adherence, the induction of cytoskeletal alterations and differential signaling activity of the various surface molecule. **Methods and Results:** The virulence of *P. brasiliensis* can be attenuated or lost after long periods of repeated sub culturing and reestablished after animal inoculation. The sample recently isolated of animals (18b) demonstrated a greater capacity to adhere, to invade the epithelial cells and also had higher levels of protein expression, when compared with the 18a. A protein of 30 kDa, pI 4.9 was more evidenced in the *P. brasiliensis* 18b extract and had an adhesin characteristic. Gp43 and 30kDa proteins seemed to be involved in specific interactions with some ECM proteins. The role of 30kDa and gp43 on cellular interactions were investigated and the adhesion of *P. brasiliensis* yeast cells was intensively inhibited by pre-treatment of epithelial cells with 30kDa protein and gp43. Cytoskeleton components of the host cells, like actin, tubulin and cytokeratin were involved in *P. brasiliensis* invasion process. Through an immunoblot assay, some proteins were also recognized by anti-actin, anti-cytokeratin and anti a-actinin sera, but not by anti-tubulin. In this study, the involvement of protein kinases (PKs) has been investigated in the interaction of *P. brasiliensis* and epithelial cells. PK inhibitor genistein could reduce cell invasion by P.brasiliensis significantly. The apoptosis induced by this fungus and these adhesins in infected epithelial cells was demonstrated by various techniques: TUNEL, DNA fragmentation and Bak and Bcl-2 immunocytochemical expression. 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.

Supported by: FAPESP; CNPq and PADC-FCF.