Round Tables
MODULATION OF THE ANTIGEN-SPECIFIC RESPONSES IN PATIENTS WITH PARACOCCIDIOIDOMYCOSIS

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Paracoccidioides brasiliensis, a thermo-dimorphic fungus, is the etiologic agent of paracoccidioidomycosis (PCM). The recrudece 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 constantly present in all patients and the percentage of CD28 negative cells is increased in patients. Induced expression 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 pursuit to dissect such mechanisms we have shown, on one hand, that stimulation with the major P. brasiliensis antigen (gp43) of PBMC from patients with active PCM lead to decreased IL-12, IL-2 and IFN-γ secretion but high IL-10 levels. In parallel, gp43 induced low (or not at all) IL-12/IL-2 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 P. brasiliensis modulates host 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 stimulated T-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.

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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 h) from patients with pulmonary PCM when compared to the levels found in healthy controls. IL-4 was not detected in this study. Th2 cytokines levels were increased in all patients compared to healthy controls. No correlation was found between HLA class II expression and cytokine levels.

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

KINETICS OF CYTOKINES AND CHEMOKINES GENE EXPRESSION DISTINGUISHES PARACOCCIDIOIDIOSE BRASILENSIS 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 demonstrated that PBMC from JF patients stimulated with Pb antigen produce higher levels of Th2 cytokines IL-4, IL-5, IL-10, and TGF-β1 than 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-10, CXCL9 and CXCL10 mRNA expression was analyzed by a semi-quantitative RT-PCR (normalized by b-actin mRNA). Protein expression was assayed by flow-cytometry. PI individuals expressed earlier and higher levels of IFN-γ, TNF-α, CXCL9 and CXCL10 mRNA which compared to JF patients. In relation to AF patients the PI group presented 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 therapeutic approach associated with antifungal drugs as new alternatives for the treatment of disseminated forms, particularly in immunosuppressed patients.
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 during the infection. Expression of TGF-B and TNF-alpha 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 plasmodocytes. 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-g 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 subsequent 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 plasmodocytes. 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 PARACOCIDIIOIDES 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 defines 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 Pb1. Methods and Results: We used the susceptible B10.A mouse model preliminarily inoculated by the intraperitoneal (i.p) route with variable-adapted cultures. The following infections were carried out by the intratracheal (i.t) and intravaneous (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 from the lungs and a pattern of Th2-driven anti-gp43 immunoglobulins (IgG1 and IgG). 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 PhGP43 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 PhGP43 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
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Paracoccidioidomyositis 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-idiotype 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 of Ab2β2 are species-independent, and mouse Ab2β2 is potentially suitable as a surrogate antigen in human PCM patients, in the present work it was also analyzed whether Ab2β2 can induce T cell immunity. Our results showed that when mice were immunized with Ab2β2 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 Ab2β2. It is tempting to speculate that Ab2β2 bearing internal image of gp43 could contain T cell epitope, which was responsible to induce lymphoproliferation. 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 Ab2β2 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.

OPPORTUNISTIC PARACOCIDIOIDOMYCOSIS
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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 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 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 lesions but 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 the estimated 1.3% prevalence of paracoccidioidomycosis in young 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 ± 45.2 years), but no significant difference was observed regarding gender (men: 81% ± 91%) or ethnic group (whites: 68% ± 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% ± 77%). Many co-infected patients had a number of circulating TCD4 lymphocytes lower than 100/mm³. Regarding the clinical signs and symptoms, HIV-1-infected patients usually had more disseminated and rapidly progressing lesions accompanied by fever (81% ± 39%). They had more skin lesions (60% ± 39%), generalized adenomegaly (42% ± 31%), hepatomegaly (55% ± 13%), splenomegaly (19% ± 4%) and pulmonary infiltrates (74% ± 62%). Dysphagia was less frequent in co-infected patients (13% ± 36%), as also was hoarseness (22% ± 20%) and lesion of the oral mucosa (21% ± 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 GP3 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 samples, 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 97.5% 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 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 during 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.

Cotrimoxazol [CMX] (960 or 1,240 mg IV or PO every 12 hrs), amphotericin B deoxychoylate [AMBd] (total dose up to 30 mg/kg), ketokonazol [KTZ] (200 or 400 mg PO every day), and itraconazol [ITZ] (100 or 200 mg PO every day) are the recommended antifungal drugs. Indications, contra-indications, efficacy, effectiveness, and side effects are well known. CMX and ITZ are the main drugs of choice and AMBd is indicated only in life threatening disseminated form. 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 (IDDI) 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 IDDI. 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.

References


DIAGNOSIS OF NEUROPARACOCCIDIOMYCOsis 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 paracoccidioidomycosis (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. MAbs anti-gp43 and Mab anti-gp70 were a gift from R. Puccia and JD Lopes, Immuno-ELISA was performed.
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 had 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 *Dasyus 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 (2), Ceará (8), 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, Paraiba and Rio Grande do Norte with similar climatic conditions as observed in Piauí.

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

References


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 evaluating cell immunity during murine histoplasmosis. CFAgs induced a more potent DTH response 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.
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 act as infectious propagules upon inhalation into the lungs, where they transform to the pathogenic yeast form.

Four distinct morphological forms of the fungus are apparent in the transition and which we term mycelium, differentiating mycelium, forming yeast and yeast. After inducing the morphological switch by increasing the incubation temperature of mycelium from 26°C to 37°C, the onset of mycelial differentiation occurs after about 12 hours, with most mycelia undergoing differentiation by 24 hours; whilst transforming yeast appear after 48 hours and reach 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 thermomorphogenic 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 °C. 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.

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**GENES EXPRESSED IN THE PATHOGENIC PHASE OF **

**PARACOCIDIOIDES BRASILIENSIS AND THEIR POTENTIAL INVOLVEMENT IN VIRULENCE AND MORPHOLOGY**

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Paracoccidioides brasiliensis, a thermophilic fungal 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.

**TRANSCRIPTIONAL PROFILES OF THE HUMAN PATHOGENIC FUNGUS PARACOCIDIOIDES 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, 253-241, 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 en 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, Ca2+/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 evidenced 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.

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MR6 - CELLULAR BIOLOGY OF Paracoccidioides brasilensis

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

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Paracoccidioides brasiliensis is a dimorphic fungus alternating between the mycelial form found at room temperature and the yeast phase detected in infected tissues or in cultures at 37°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. Immuno proteomic approaches allowed the characterization of molecules such as a 60-kDa heat shock protein (PbHSP60) and two enzymes of the glycolytic pathway, glyceraldehydes 3-phosphate dehydrogenase (PbGPADPH) and triose phosphate isomerase (PbTPI). All molecules are preferentially expressed in the yeast parasitic phase and the transcripts and protein levels are accumulated during the mycelium to yeast transition. PbGPADPH and PbTPI 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 extracellular matrix proteins, such as laminin and fibronectin. The recombinant purified proteins, rGPADPH 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 PbHSP60 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 PbGPADPH and PbTPI 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 PbHSP60, 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.

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PARACOCIDIOIDES BRASILIENSIS: INTRA OR EXTRACELLULAR FUNGUS?
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There are direct and indirect evidences that Phialophora 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 by cultures 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- and arterial 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 (invasivation) 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 PARACOCIDIOIDES 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, fibrogen and laminin were bound to the surface of conidia and to a lesser extent, also to myocardial 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, fibrogen and laminin, as the same proteins when added in soluble form, except in the case of fibrogen. This interaction was inhibited by the presence of the fibrogen degradation fragment D. Various monosaccharides and the peptides RGD and RGDG has had no effect on adherence of conidia to immobilized ECM proteins. However, N-acetylnleuranamic 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, fibrogen and laminin. Two polypeptides were detected in both fungal forms and their corresponding SDS extracts were shown to interact 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 mononclonal 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, indicates the presence of common receptors for laminin, fibronectin, and fibrogen.

These fungi 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- and arterial 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 (invasivation) 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.
EFFECTS OF NEW AND AN OLD ADHESINS IN CYTOSKELETON, APOPTOSIS AND SIGNALING. ADHESION AND INVASION OF PARACOCIDIOIDES 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 α-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.

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