CONFERENCES

Conference 1

THE POWER OF THE SMALL: Paracoccidioides brasiliensis CONIDIA

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The beginning (1972). For many years research on *Paracoccidioides brasiliensis* (*Pb*) centered in the yeast (Y) leaving aside the mycelium, probably because its lack of distinctive markers. Few authors, Borelli (1955) and Pollak (1971) among them, accurately described the propagules produced by this form. In 1972 the serendipitous observation of conidia (C) led - a decade later - to a series of research studies, aimed at determining their more relevant characteristics. At the beginning, the main difficulty was the fungus' scanty sporulation but and after some trials, the problem was partially solved, thus opening the way to a series of experimental studies that demonstrated the outstanding capacities of this small (3.9-4.4 µmicrons) but resourceful fungal propagule.

The initial developments (1985-1991). C formation was shown to be a late event occurring after prolonged (2-3 months) incubation and only when the mycelium was grown in lean media, such as water agar. Slide culture observations of undisturbed mycelial growth were used to microscopically determine C types, finding intercalary arthroconidia, C stemming from the latter, and single-celled, pedunculated C. Because arthroconidia were an integral part of the parent mycelium their detachment proved difficult and it was necessary to design a method for recovering single units, needed for further testing. Saline wetting of the mycelial surface with careful scraping produced a suspension that was shaken with glass beads, centrifuged and filtered through glass wool; C were then counted and their viability assessed (ethidium bromide). Yield was approximately 1000 C/plate. Electron microscopic studies revealed that they possessed all the attributes of viable and physiological competent eukaryotic cells. In mature C the cytoplasm was densely packed with food reserves including lipid bodies, indicating that these propagules were well provided for surviving environmental stress. The capacity of the conidium to respond to temperature changes was then tested; slide cultures were prepared for microscopic examination and incubated at 22°C or at 36°C. In the first case, C began to produce germ tubes in 24h giving rise to mycelial elements after 96 h; when at body temperature; they rounded up and in 72-96h converted into Y cells some of which exhibited the multiple budding configuration. Going from 4 µm to 40 µm in such short time was, indeed, a wonder. This finding was accompanied by observations showing that 80% of the uninucleated C incubated at 37°C became multinucleated when reaching the Y stage. The time had come to determine the infectiousness of *Pb* propagules for BALB/c mice. In 1985 it was difficult to obtain large numbers of C for animal inoculation and a modest number ($1x10^6$) had to be used for intranasal inoculation. Even so it was possible to observe that the C reached the alveoli and began to transform into Y in 12 h; by 18 h multiple-budding Y cells had already appeared. The rate of pulmonary infection was 98%. Inoculation was followed by an intense inflammatory reaction leading to granuloma formation and to increased numbers of actively multiplying Y cells. In a few days dissemination took place form the lungs to the liver and spleen. Thus C appeared capable of producing active disease in healthy animals. Under identical experimental circumstances Y were less virulent needing a much larger inoculum ($20x\ 10^6$) and causing lesser organ damage. Little wonders!

The present (1992-2001). The above experiment was quite revealing and indicated that other studies could be undertaken with the natural infectious propagule. Several research lines were delineated during this period. One centered on the host-parasite interactions via phagocytosis finding that macrophages were permissive to conidia and were killed only if they were activated. Other explored the *in vitro* and *in vivo* influences of human hormones on the adaptation of conidia to host tissues observing that males developed a severely disseminated disease while females controlled the infection. Another attempted to reproduce the fibrotic sequelae observed in human paracoccidioidomycosis, observing that granulomas were the center of activity with collagen fibers surrounding them and fibrosis beginning 8-12 weeks post-challenge. An observation related to the dark coloration noticed when conidia were suspended in saline, led to elegant and thorough studies that clearly revealed that C were indeed melanized.

The outcome (2002). Research grows little by little and fulfills its goals only when the joy of understanding is shared. It has been so with *Pb* small propagules.

Conference 2

THE DIMORPHIC TRANSITION IN Paracoccidioides brasiliensis, A MATHEMATICAL AND PHYSICO-CHEMICAL CHALLENGE

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Fungal dimorphism is related to pathogenesis, as only one of the forms is usually associated with the disease. The term dimorphism implies two distinct morphological phases, whose conversion from one to the other is triggered by environmental stimuli. But many intermediate cell shapes occur frequently. Merson-Davies & Odds (1989) quantified morphological forms of *Candida albicans* to facilitate definition of cellular and molecular markers specific for cell development, and determine the distribution of morphological types in different environmental conditions. By relating their maximum length, and maximum and septal diameters in a mathematical relationship, a morphology index (Mi) was established, ranging from 1 in spherical yeast cells to 4 in true hyphae, with elongated yeast cells and pseudohyphae giving intermediate values. In this way, the subjective descriptions of cell morphologies were substituted by objective parameters aimed to facilitate comparison of inter-laboratory data and a better distinction between phenotypes. Following this example, morphological forms (extreme and transitional) of *P. brasiliensis* were mathematically quantified. A morphological index (Mi) was defined by maximum cell length (l), maximum cell diameter (d), and septal diameter (s): $Mi = 2.13 + 1.13 \log_{10} (ls/d^2)$. Intercept and slope were such that Mi was 1 for yeast (Y) or 4 for mycelia (M). This discriminatory power was used to quantitate morphological population mixtures through Mi histograms. During transition (either way), average Mi (Mi) varied linearly with time, suggesting a continuity in the process. Also, an inverse relationship between Mi and content of both cell wall chitin and alpha-1,3-glucan was found (San-Blas *et al.*, 1997).

A second approach was used to focus on the physico-chemical modifications of *P. brasiliensis* cell walls through the dimorphic transition. For this, molecular modeling was used. Though a well-validated method for the study of polymers, no work has been done on fungal wall biopolymers. Molecular mechanics provides information about the most stable conformation of molecules and their aggregates, making use of analytical functions to represent bond stretching, bending, and torsional as well as nonbonded (electrostatic interactions, dispersion attraction and exchange repulsion) energies of molecules. In this way, an initial configuration is specified and the interatomic distances and bond angles are adjusted, using an iterative computational method, until the minimum energy configuration is obtained. The algorithms used in this work were part of the INSIGHTII and DISCOVER set of programs. To obtain the most stable conformation for the molecules and aggregates, it is necessary to use procedures that include molecular dynamics. In this way, the system may surmount energy barriers that lead to more stable molecular (or aggregate) conformations. Molecular dynamics and mechanics were used to study the *P. brasiliensis* cell wall glucans in its two morphologies (beta-1,3-glucan in the M phase and alpha-1,3-glucan in the Y phase). Strand morphologies from both polymers were dissimilar. Intramolecular correlation functions (CF) were almost identical indicating that the short order is conserved. Intermolecular CF were different, hence the conformation of the polymer (alpha or beta) is relevant to the resulting disorder of their solid phase and will have implications in the shaping of the fungal cell by changing its mechanical properties.

References:
Merson-Davies, L.A., Odds, F. C. (1989). *J. Gen Microbiol.* 135: 3143-3152.
San-Blas, G., Padrón, R., Alamo, L, San-Blas. F. (1997) *Microbiology* 143: 197-202.

Supported by Grant G-97-000615, FONACIT (Fondo Nacional de Ciencia y Tecnología), Caracas

Conference 3

SIGNALLING MECHANISMS THAT CONTROL HYPHAL GROWTH AND VIRULENCE IN THE PATHOGENIC FUNGUS Candida albicans

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The human fungal pathogen *Candida albicans* switches from a budding yeast form to a polarized hyphal form in response to various external signals. This morphogenetic switching has been implicated in the development of pathogenicity. We demonstrate through the functional characterization of the *C. albicans* homologs of adenylate cyclase (CaCdc35) (Rocha et al.: Mol. Biol. Cell 12, 3631-3643, 2001) and the small G-protein Ras (CaRas1) (Leberer et al.: Mol. Microbiol. 42, 673-687, 2001) that a Ras/cAMP regulated signal transduction pathway plays a role in morphogenetic switching and virulence of this fungus. Epistasis experiments demonstrate that the Ras/cAMP pathway induces the induction of hyphal specific genes through activation of the putative transcription factor Efg1. Moreover, these epistasis experiments indicate that this pathway is tigthly coordinated with a previously characterized filament-inducing MAP kinase cascade that regulates the transcription factor Cph1 (Leberer et al.: PNAS 93, 13217-13222, 1996). We propose that signalling networks including Ras/cAMP and MAP kinase mediated mechanisms trigger morphological switching of *C. albicans* and thereby contribute to the pathogenicity of this dimorphic fungus. Thus, components of signalling pathways may be valid targets for the development of new therapeutic antifungal strategies.

Conference 4

NEW ANTIFUNGAL DRUGS UNDER CLINICAL INVESTIGATION

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Nowadays we are facing a new epidemiologic scenario where opportunistic fungal infections represent a major problem in tertiary care hospitals worldwide. These infections are difficult to diagnose and some of them are refractory to antifungal therapy. *Candida* spp and *Aspergillus* spp respond for more than 90% of all nosocomial fungal infections and both are important target microorganisms for new antifungal drugs. Regarding to *Candida* spp infections, the emergence of systemic infections due to non-*albicans Candida* species is clearly a concern. Recently, fluconazole resistant *Candida* isolates have been reported, mostly related to AIDS patients under fluconazole long term therapy. Systemic mycosis due to *Trichosporon* spp, *Fusarium* spp and *C. lusitaniae* have been recognized by different centers as infections refractory to amphotericin B. In contrast with the increasing number of pathogens causing systemic fungal infections we have only 5 drugs for antifungal therapy of invasive mycoses. In this scenario, amphotericin B is still considered the gold standard therapy for most of all invasive infections despite the serious adverse effects it may cause to the patient. It is clear that we need more potent and safety antifungal drugs to better treat our patients with fungal infections.

Currently, we have 6 new antifungal drugs under advanced stage of clinical investigation, including 3 new triazoles and 3 compounds that belong to a new class of antifungal drugs, the equinocandins. The triazoles of second generation include ravuconazole (BMS207147), posaconazole (SCH56592) and voriconazole. The mechanism of action of all these new triazoles is the inhibition of the cytocrome P450 dependent enzyme 14αdemethylase (14αDM). The overexpression of CDR genes (ABC transporters related to efflux systems that pump drug from the cell) is considered a major mechanism of resistance among *C. albicans* and non-*albicans* isolates and may cause cross resistance with different triazoles. However, most of all isolates of *Candida* spp, including *C, glabrata* and *C krusei*, are very susceptible to the mentioned azoles. In addition, these compounds are very active *in vitro* and in animal models against *Cryptococcus* spp, *Aspergillus* spp, *Trichosporon* spp and agents of endemic mycosis, including *P. brasilienis*. Voriconazole (oral and intravenous formulation) is the new triazole which has more clinical data available. This drug has been evaluated in large clinical trials of patients with neutropenia and persistent fever, esophageal candidiasis as well as in invasive aspergillosis . Data obtained from these studies illustrated that voriconazole is a safe antifungal drug with good clinical activity against *Candida* and *Aspergillus* infections. Additionally, there are some *in vitro* and clinical data suggesting that voriconazole may have antifungal activity against some *Fusarium* spp and *Scedosporium* spp isolates.

Echinocandins are a new class of fungicidal drugs that inhibit the synthesis of β-(1,3)-D-glucan, thus interfering with fungal wall synthesis. There are 3 equinocandins under clinical investigation: caspofungin, micafungin (FK463) and LY303366. Pre-clinical studies have demonstrated that all these drugs have very good activity against *Candida* spp and *Aspergillus* spp and appear to have some activity against *H. capsulatum* and *C. immitis*. Caspofungin (IV formulation) is the echinocandin with more clinical data available and its clinical use for invasive aspergillosis was already approved by the FDA-USA. There are also clinical data suggesting that caspofungin has very good activity against *Candida* invasive infections. No clinical reports of acquired echinocandin

resistance have been published but mutations at FKS1(gene that encodes a subunit of 1,3-β-Glucan synthase) is a potential mechanism for developing resistant isolates.

Conference 5

MELANIZATION OF PATHOGENIC FUNGI

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Melanin is made by several important pathogenic fungi and has been implicated in the pathogenesis of a number of fungal infections. Melanins are high molecular weight, negatively charged, hydrophobic pigments that are formed by the oxidative polymerization of phenolic and/or indolic compounds. Melanins are found in species from all biological kingdoms. We developed methods for the isolation of melanin from pathogenic fungi utilizing proteolytic enzymes, denaturant, phenol/chloroform extractions, and hot acid. We applied these protocols to determine whether melanin or melanin-like compounds are produced *in vitro* and during infection in *Cryptococcus neoformans, Paracoccidioides brasiliensis,* and *Histoplasma capsulatum.* The yeast forms of the fungi require the presence of phenolic substrate to produce melanin. Conidia from the dimorphic fungi do not require exogenous substrate to synthesize melanin. Treatment of pigmented cells with enzymes, denaturant, and hot concentrated acid yields dark particles similar in size and shape to their respective propagules. The melanins isolated from the fungi react with a melanin-binding mAb developed to *C. neoformans* melanin. Electron spin resonance spectroscopy reveals that pigmented yeast cells and particles derived from pigmented cells contain stable free radicals consistent with their identification as melanins. The melanin-binding mAb labels yeast cells in tissues from infected mice or from biopsy specimens from infected patients. Digestion of infected mouse tissues yields dark particles that react with the melanin-binding mAb and are similar in appearance to the yeast cells. Additionally, sera from infected mice contain antibodies that bind melanin particles. Phenoloxidase activity capable of synthesizing melanin from L-DOPA is detected in cytoplasmic yeast cell extracts. Furthermore, we have also confirmed that *Sporothrix schenckii* conidia produce melanin and have also demonstrated melanization of yeast forms *in vitro.* We have also demonstrated that inhibition of melanogenesis in *C. neoformans* by the administration of glyphosate (an herbicide that inhibits the shikimate pathway) or melanin-binding mAbs significantly increases survival of infected mice. In summary, we have demonstrated that *C. neoformans* yeast cells and that both the conidial and yeast forms of *P. brasiliensis* and *H. capsulatum* can produce melanin or melanin-like compounds *in vitro* and that yeast cells can synthesize pigment *in vivo.* This work suggests that the protective mechanisms attributed to melanin in *C.* neoformans *in vitro* also apply *in vivo.* Based on what is known about the function of melanin in the virulence of *C. neoformans* and other pathogenic fungi, this pigment may play a similar role in the pathogenesis of paracoccidioidomycosis, histoplasmosis, and sporotrichosis.

Conference 6

GENETIC TOOLS TO STUDY THE PATHOGENESIS OF Blastomyces dermatitidis

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The dimorphic fungal pathogen *Blastomyces dermatitidis* exists as a sporulating mold in soil throughout the eastern United States, typically infecting human and animal hosts when its habitat is disrupted and conidia become airborne. Upon introduction into a host the fungus undergoes a temperature induced phase transition to the pathogenic yeast form and initiates production of a 120-kD protein designated Blastomyces Adhesin 1 (BAD1, previously WI-1). BAD1 promotes adherence of the yeast to macrophages through binding of CR3 receptors. Both the BAD1 adhesin and the phase transition are indispensable in the establishment of pulmonary infection and the subsequent dissemination of the yeast to other organs, skin and bone.

Several recent advances in the molecular tools for genetic manipulation of *Blastomyces dermatitidis* have aided in the investigation of these components of pathogenesis. Here, we will describe successes in gene targeting, new selection markers, advances in gene-transfer technique, and reliable reporter fusions. These advances come at

an opportune time, as an improved understanding of pathogenic mechanisms is needed to further research into therapeutic options for Blastomycosis and similar diseases worldwide.

Using an electric-pulse protocol to introduce exogenous DNA, our laboratory recently engineered a strain of *B. dermatitidis* in which the BAD1 genetic locus was disrupted by gene replacement. Strain #55 is devoid of BAD1 protein, but is otherwise viable and morphologically similar to the parent strain, ATCC 26199. Strain #55 nevertheless binds poorly to macrophages *in vitro* and to lung tissue *ex vivo,* and is avirulent in a murine model of pulmonary infection. These properties were fully restored after reconstitution of BAD1 by means of gene transfer, establishing the pivotal role of BAD1 in adherence and virulence of *B. dermatitidis* yeast. To our knowledge, this was the first example of a genetically proven virulence determinant among systemic dimorphic fungi.

The gene for chlorimuron ethyl resistance from *Magnaporthe grisea* has been discovered to be particularly useful in transformation of *B. dermatitidis*, and was instrumental in re-transforming the previously manipulated, hygromycin-resistant strain #55. The G418 resistance gene has been successfully employed as well, and newly developed auxotrophic strains give us further options for genetically manipulating these organisms. For years, prior to the development of these selection strategies, hygromycin resistance remained the sole means of transformant selection.

Agrobacterium tumefaciens-mediated gene transfer, developed originally for the transformation of plant species, has been adapted for use with *B. dermatitidis.* This new method for generating transformants has shown a greatly increased frequency of transformation: up to 10^3 per 10^7 target yeast. This technique has recently been used to insert beta-galactosidase (LacZ) reporter genes fused to the BAD1 promoter region into North American *B. dermatitidis,* African *B. dermatitidis, and Histoplasma capsulatum* (all dimorphic fungi), allowing an in depth study of the conservation of phase-specific transcription.

Conference 7

IMMUNOPROTECTIVE MACROMOLECULES OF Coccidioides posadasii

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The evaluation of recombinant proteins of *Coccidioides posadasii* for their protective efficacy in a murine model of coccidioidomycosis has revealed several potential vaccine candidates. Two of the most promising of these are a recombinant proline-rich antigen (rAg2/Pra) and a glucanosyltransferase (Gel1). Both antigens are associated with the cell wall as a result of glycosylphosphatidylinositol-(GPI) linkages, either to the plasma membrane (Ag2/Pra) or to glucan components of the wall matrix (Gel1). A homolog of Ag2/Pra has recently been isolated from *C. posadasii* which shows high sequence identity to the previously described, immunoprotective antigen. Although this newly discovered antigen (Pra2) is also proline rich, it lacks the four amino acid repeat which is characteristic of Ag2/Pra. It also lacks the GPI anchor and is apparently secreted from the parasitic cells. The ideal vaccine against coccidioidomycosis is considered to be one which activates a Th1 pathway of immune response, stimulates cellular immunity to different stages of the parasitic cycle, and contributes to host clearance of the pathogen from sites of infection. Our current studies involve the evaluation of the protective efficacy of combinations of Ag2/Pra, Gel1, and Pra2. Our hypothesis is that these antigens in combination are more effective in stimulation of protective immunity than the single antigens. Our evaluations of the response of vaccinated C57BL/6 mice to *C. posadasii* infection includes ELISA assays of cytokine proteins present in bronchoalveolar lavage fluid of infected immune and control mice, in situ examination of cytokine gene expression by laser capture microdissection and real-time polymerase chain reaction, fungal burden determinations at different times post-challenge, and survival analysis. The principal goal of these investigations is to develop a vaccine against coccidioidal infection which can provide protection to the heterogeneous human population exposed to this primary fungal pathogen.