ROUND TABLES

Round table 1

A GENOMIC APPROACH TO VIRULENCE IN Candida albicans

Magee, P.T.; Forche, A.; Chibana, H.; Lephart, P. and Magee, B.B.

Department of Genetics, Cell Biology, and Development, University of Minnesota, St. Paul, MN USA.
E-mail: ptm@cbs.umn.edu

Candida albicans, the major human fungal pathogen, is a diploid organism as usually isolated. For the past several years, efforts have been ongoing to determine the complete genomic DNA sequence and to devise a physical map of the eight chromosomes. In 1998 a complete physical map of the smallest chromosome, chromosome 7, was published. In the year 2000, sequence amounting to about 168 million bases, or 10.5-fold coverage of the genome, was released. Because of the diploid nature of the genome and extensive heterozygosity, assembly of the sequence has been difficult. However, a final diploid assembly is close to release. *C. albicans* has a highly variable karyotype and the information provided by the genome project (sequencing and mapping) has yielded insight into the interesting genome structure of *C. albicans*, and the mechanisms by which karyotypic variation occurs. In particular, a middle repeat sequence called the Major Repeat Sequence (MRS), originally studied in the laboratory of K. Tanaka (Iwaguchi, et al, 1992. J. Gen. Microbiol. 138: 1893), seems to be involved in chromosome length polymorphism, in translocations, and in chromosome loss by non-disjunction. The relationship of this variability to pathogenesis is not clear, but karyotypic variants are found much more frequently in clinical isolates than in laboratory strains. In order to determine the rate at which karyotypic rearrangements occur *in vivo*, we have used a gal1/GAL1 heterozygote to monitor mitotic recombination *in vivo* and have developed a microarray method to examine recombination involving unselected markers.

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USES OF GENOMIC INFORMATION: MATING IN THE "ASEXUAL" YEAST Candida albicans

Magee, B.B.; Legrand, M. and Magee, P.T.

Department of Genetics, Cell Biology, and Development
University of Minnesota, St. Paul, MN USA.
E-mail: Bebe@cbs.umn.edu

Determination of the complete genomic DNA sequence of the diploid and presumably asexual *Candida albicans* led to the identification of a gene highly homologous to the *MAT***a** gene in *Saccharomyces cerevisiae*. Further exploration of this locus (Hull & Johnson, 1999) led to the identification of a complete mating type region, with both sexes (**a** and alpha) represented, each on one homologue of a particular chromosome. Subsequently, two laboratories induced mating in *C. albicans*, one by deleting particular genes of the *MTL* (Mating-Type Locus) (Hull et al, 2000) and one by inducing loss of one or the other homologue of chromosome 5, the site of the *MTL* (Magee and Magee, 2000). Mating *in vitro* was shown to require low temperature, extended periods of time, and large cell numbers. Analysis of cells with disruptions in homologues of several of the genes involved in the pheromone response pathway in *S. cerevisiae* showed that this pathway is required for mating in *C. albicans* as well. Although no mating pheromones have been identified in *C. albicans*, homologues of genes involved in pheromone processing have been shown to be required for the process, suggesting that the pheromones are very similar in the two distantly related yeasts. A genomic search has identified several candidate open reading frames for Candida mating pheromones. Microscopy reveals mating figures in *C. albicans* which are morphologically dissimilar to those in *S. cerevisiae*.

References: Hull, C.M. & Johnson, A.D. (1999) *Science* **285**, 1271-5.
Hull et al (2000) *Science* **289**, 307-310.
Magee, B.B. & Magee, P.T. (2000) *Science* **289**, 310-3.

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THE Paracoccidioides brasiliensis EST GENOME PROJECT

Goldman, G.H.

Faculdade de Ciências Farmacêuticas de Ribeirão preto, Universidade de São Paulo, Brazil.
E-mail: ggoldman@usp.br

P. brasiliensis is the etiological agent of paracoccidioidomycosis - a systemic life-threatening mycosis affecting the rural population of Central and South America. Despite the importance of the fungus, little is known about its cell and molecular biology or the processes critical for its growth, morphogenesis, and pathogenesis that might be exploited in chemotherapeutic research. Genomic technologies enable the identification of complete gene sets that control pathways such as those leading to pathogenicity. Expressed Sequence Tags (ESTs), single pass reads from randomly selected cDNA clones, have proved to be a valuable resource for genome research. The use of large EST data sets allows the analysis of many thousands of genes simultaneously. We have decided to construct a *P. brasiliensis* cDNA library from the yeast pathogenic form and sequenced 13,490 ESTs from both 5' and 3'-ends. Cluster analysis using the CAP3 program showed 3,295 singlets and 1,397 contigs. Sequence analysis of these clusters have already identified among them several virulence factors present in other human pathogenic fungi, such as *Candida albicans* RBT2 and RBT5 genes that encode a ferric reductase and a secreted cell wall protein, respectively. We have also identified several cDNAs that correspond to genes encoding novel cell wall proline-rich proteins and adhesins. Our project provides a frame for further progress into the biology of this important human pathogen.

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FUNCTIONAL AND DIFFERENTIAL GENOME PROJECT OF *Paracoccidioides Brasiliensis*

Felipe, M.S.S. and Pbgenome Network.

Laboratório de Biologia Molecular - Instituto de Ciências Biológicas – Universidade de Brasília - DF. Brazil. CEP: 70.910.900.
E-mail: msueli@unb.br

Paracoccidioides brasiliensis is a dimorphic fungus, which occurs in two forms; mycelium at 26°C and yeast form at 36°C. This fungus is the etiological agent of the paracoccidioidomycosis, a systemic mycosis having restricted geographical distribution in Latin America, where affects specially rural workers and patients with immunological deficiency. It is important to point out that the establishment of the infection is dependent on the mycelium to yeast transition. This work represents the initial stage of the general project "Functional and Differential Genome of *P. brasiliensis*" and the main goals are the identification of expressed genes in mycelium and yeast forms by ESTs (expressed sequence tags) sequencing, generating information about the transcriptome of *P. brasiliensis*. As a consequence, the differential genes expressed in yeast and mycelium cells will be identified by electronic subtraction and/or microarray. We have performed standardization protocol for the production of ESTs using cDNA libraries from the mycelium and yeast cells; sequencing and computational analysis as well as the annotation and inference of possible gene function. A total of 3.938 ESTs (Y= 1.654 and M= 2.274) were generated, forming 1.563 *singlets* (Y= 603 and M= 960) and 597 *contigs* (Y plus M). After similarity analysis in database (BLASTx), 894 clusters using COG were annotated in 18 functional categories. Genes that code for members of the Heat Shock Proteins family (mycelium: HSP 82, HSP 10, HSP 30 and HSP 88; yeast: HSP 70, HSP 60 and HSP 1; in both: GroEL) were identified which could potentially be involved in the dimorphic and thermo regulated transition of *P. brasiliensis*. We have also identified many other interesting genes such as: a) Prohibitin, which exhibits anti-prolipherative activities, controlling senescence process and is also involved in the maintenance and regulation of mitochondrial morphology; b) Polyubiquitin, which may be involved either in the dimorphic transition or in the differentiation process of this fungus; c) Multi-drug resistance proteins (MDR) which might help in the design of new drugs for infection control and the elimination of this fungus; d) Proteins involved in meiotic sister-chromatid recombination (Msc1p). Functional analysis will result in important information about expression, cellular differentiation and pathogenicity and/or virulence of *P. brasiliensis*, but this issues will only be further addressed as gene-disruption and/or RNA interference (siRNA) approaches become available for this pathogen.
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Round table 2

PbrChs4: A NEW CLASS OF FUNGAL CHITIN SYNTHASE?

Niño-Vega, G.; Carrero, L. and San-Blas, G.

Instituto Venezolano de Investigaciones Científicas, Apartado 21827, Caracas 1020A, Venezuela.
E-mail: gnino@ivic.ve

Two characteristics makes the fungal cell wall a good target for the development of antifungal antibiotics: its integrity is essential for the survival of the fungus, and it is mainly composed of polysaccharides which are not found in animal cells. As chitin is a major component in the cell wall of the pathogenic yeast form in *Paracoccidioides brasiliensis*, and its content is almost three times higher than in the mycelial cell wall, its biosynthesis is an attractive target for the use of antifungals antibiotics against this medically important fungus in Central and South America.

In *P. brasiliensis*, five different chitin synthase genes have been so far identified (Niño-Vega, G.A., *et al.*, 2000). One of them, *PbrCHS2* (codifying for a putative class II chitin synthase) has been cloned (Niño-Vega, G. A., *et al.*, 1998). In the present study, we present the cloning of PbrCHS4, which codifies for a putative class V chitin synthase. Sequence analysis of the deduced protein suggests the presence of two domains: a C-terminal domain, with high homology to class V chitin synthases ranging from 41% to 56% in around 1070 aa, and an N-terminal domain, with low homology to myosin motor domains (between 26% and 30% in 300 aa). However, comparisons of the N-terminal domain of PbrChs4 to the same region of class V chitin synthases with myosin motor-like domains, reveals an even lower homology (between 25% and 29% in less than 300 aa). So far, homologies reported at the N-terminal region of class V chitin synthases with myosin motor-like domains are as high as 65.8% in 700 aa, and around 60% in 1500 aa for the chitin synthase C-terminal domain (Park, I.C., *et al.*, 1999). Based on these results, we suggest that PbrChs4 may be classified at least as a subclass of the classV chitin synthases.

References:
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PROTEASES AND CATALASES OF Paracoccidioides brasiliensis

Soares, C.M.A.

Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás, 74001-970-Goiânia-Goiás-Brazil.
E-mail: celia@icb2.ufg.br

Paracoccidioides brasiliensis is the etiological agent of paracoccidioidomycosis (PCM) a fungal disease that affects many individuals in Latin America. The mycelia found in nature constitute the infective phase that differentiate to the yeast form in the human lungs establishing the infection. The fungus is an active participant in the infective process and some putative virulence factors have been described. Here we report the characterization of sequences encoding catalase and a Heat Shock Protein (HSP) member of the ClpB sub-family of *P. brasiliensis*. Catalases play a key role as antioxidant, protecting aerobic organisms from the toxic effects of hydrogen peroxide. Increasingly evidences support the contention that endogenously produced fungal catalases might abrogate the effects of the host oxidative fungicidal mechanisms, acting as virulence factors. In addition, catalases are described as immunodominant antigens in several microrganisms. Proteases of the Clp family possesses chaperon like attributes and had been shown to play a role in virulence of microrganisms. An antigenic catalase of *P. brasiliensis*, molecular mass of 61 kDa, pI of 6.2 had been previously characterized in our laboratory. Degenerated PCR primers were used to amplify a 690-bp product from genomic DNA of *P. brasiliensis*, ATCC MYA826. By using this product as a probe, clones were isolated from a cDNA library, presenting high degree of sequence conservation when compared to other catalase homologues. Canonical motifs characteristic of peroxisomal catalases were found. In the course of our studies attempts were made to analyze the expression of catalase in P. *brasiliensis*. One mRNA species

is preferentially expressed in the yeast fungus phase, in agreement to the levels of catalase detected in this stage of *P. brasiliensis*. The catalase levels increased early during the fungus transition from mycelium to yeast, suggesting a role for these proteins during this development in *P. brasiliensis*. *P. brasiliensis* encounter heat stress as a regular feature of its life cycle. The elevated temperature it encounters within the host serve as a key to trigger the transition from mycelium to yeast, the infective pathway. We characterized the first sequence of a putative heat shock protein, member of the ClpB sub-family of ATP dependent proteases in *P. brasiliensis*. An open reading frame of 2436-bp was identified, presenting canonical sequences of the ClpB sub-family. A transcript of 3.0 Kb and a protein species of 89 kDa, pI 5.3, were detected in the yeast parasitic phase. The ClpB sequence was also useful in the isolation of other sequences encoding related proteins, indicating the presence of a family of ATP-dependent proteases in *P. brasiliensis*.

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CHARACTERIZATION OF DIFFERENTIALLY EXPRESSED GENES IN Paracoccidioides brasiliensis

Silva Pereira, I.

Laboratório de Biologia Molecular, Departamento de Biologia Celular, UnB, Brasília-DF, Brazil.
E-mail: xocolau@unb.br

The differential expression of genes is central in several biological processes, including cell cycle, cellular differentiation, embrionary development, host-pathogen interactions and dimorphic transition. In *P. brasiliensis,* the transition from mycelium to yeast form, probably triggered by temperature shift seems to be essential for disease establishment and is easily reproduced *in vitro*. This characteristic makes *P.brasiliensis* an attractive biological model. Furthermore, the characterization of differentially expressed genes in this organism may be of great importance for understanding its life cycle, the pathogenic process, and for the development of new strategies for Paracoccidioidomycosis treatment, which is one of the most prevalent systemic mycosis in Latin America. Using various approaches to analyze differential gene expression, our group could detect several genes and/or proteins differentially expressed in *P.brasiliensis*. Here we will discuss about the isolation and characterization of a full-length mycelium specific cDNA, encoding a putative hydrophobin (*Pbhyd*). Hydrophobins are small secreted fungal proteins that play a role in a broad range of processes in the growth and development of filamentous fungi. It has been described their involvement in the formation of aerial structures, in the attachment of hyphae to hydrophobic surfaces, participation in cell wall formation, and in the establishment of infection of pathogenic fungi. Another differentially expressed gene, also identified by DDRT-PCR, was shown to be up regulated during mycelium to yeast transition. Although not yet fully characterized, this gene could be possibly implicated in the dimorphic process since its respective mRNA was detected two hours after the temperature switch from 22°C to 36°C. Presentely, we are engaged in the functional genome analysis which will greatly improve the characterization of differentially expressed genes of *P. brasiliensis*.

GENES ENCODING ENZYMES OF THE CELL WALL METABOLISM IN Paracoccidioides brasiliensis

Pereira, M.

Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás, 74001-970, Goiânia, GO, Brazil.
E-mail: mani@icb2.ufg.br

Paracoccidioides brasiliensis is a dimorphic organism that can grow as budding yeast or mycelium of filamentous hyphae. In *P. brasiliensis* both the molecular architecture and the functional components of the cell wall vary between yeast and mycelia forms of the organism. Genes that codify for enzymes involved in biosynthesis/degradation of the cell wall of *P. brasiliensis* are of great interest, since the understanding of this process can elucidate aspects of morphogenesis, invasion, infection and virulence of this fungus. Our group has been performing studies intending the isolation and characterization of cell wall metabolism related genes and its products in *P. brasiliensis*. In this vein we had characterized cDNA/genes encoding 1,3-β-D-glucan synthase, N-acetyl-β-D-glucosaminidase (NAG) and mannosyltransferase of *P. brasiliensis*, isolate *Pb*01 (ATCC MYA 826). We previously reported the cloning of characterization of the *PbFKS1* gene encoding a putative protein of 212 kDa, pI 8.5, probably

inserted in the cell membrane. Also, a cDNA encoding the NAG enzyme of *P. brasiliensis* named *Pb* NAG1 was cloned and characterized. The nucleotide sequence of the cDNA with 2663 nucleotides, presented a single open reading frame encoding a protein with a predicted molecular mass of 64.73 kDa, isoeletric point of 6.35. The predicted protein is composed of a putative 30-amino-acid signal peptide. The deduced protein shares sequence similarity to classical NAGs. The primary sequence of *Pb* NAG1 was used to infer phylogenetic relationships. The amino acid sequence of *Pb* NAG1 has 45, 31 and 30% identity to the sequences of those from *Trichoderma harzianum*, *Emericella nidulans* and *Candida albicans*, respectively. In particular, it was observed striking homology with the active site regions of the glycosyl hydrolases, family 20 proteins. The active site consensus motif G X D E and the catalytic Asp and Glu residues were found in the presently described protein (positions 373 and 374), reinforcing that it belongs to the glycosyl hydrolases family 20. Also, a sequence (*Pb*Ymnt) encoding an antigenic protein was obtained and characterized. The cloned genomic sequence identified a single open reading frame coding for a protein containing 357 amino acid residues, 39.78 kDa. The deduced amino acid sequence exhibits identity to mannosyl and glycosyltransferases from several sources. The deduced amino acid sequence contains a DXD motif, characteristic of the glycoslyltransferases. Hidropathy analysis revealed that the deduced protein presents a single transmembrane region near the amino terminus of the molecule suggesting a type II membrane protein. The *Pb*Ymnt was expressed preferentially in the yeast fungus parasitic phase. The alterations in cell wall constituents and architecture during growth and dimorphism of *P. brasiliensis*, constitute a potential target for the development of new antifungal drugs. In this context the characterization of these genes and encoding cDNAs will allow the production of the recombinant protein in order to explore potential new drugs.

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Round table 3

ECOLOGICAL AND EVOLUTIONARY ASPECTS OF Paracoccidioides brasiliensis

Bagagli, E.

Departamento de Microbiologia e Imunologia, UNESP, Campus de Botucatu, SP, Brazil.
E-mail: bagagli@ibb.unesp.br

The habitat of the mycelial saprobe phase of *Paracoccidioides brasiliensis*, which produces the infective propagula, has not been determined and has proven difficult for mycologists to describe. The fungus is rarely isolated from the environment; the disease has a prolonged latency period and no detection of outbreaks. This fact has impeded the adoption of preventive measures to avoid infection. The confirmation of natural infection of *P. brasiliensis* in armadillos, in a high frequency and wide geographic distribution, has opened new opportunities for the study and comprehension of its fungal ecology. The nine-banded armadillos have no large home ranges and the animals live in constant and restricted sites for consecutive years, which make them ideal to pinpoint the whereabouts of the fungus in nature. The armadillo's burrows and places used for animal forage encompass several favorable ecological conditions for the fungus, and probably harbor its saprobe environmental phase, as already proposed by Borelli more than 30 years ago. Ecological studies carried out in the Botucatu hyperendemic area of Paracoccidioidomycosis (PCM) has indicated that *P. brasiliensis*-positive armadillos are found most frequently in sites with disturbed vegetation, including artificial *Pinus* and *Eucalyptus* forests, and tropical riparian forests located near water sources, both having different sand-soil composition. New insights on the ecology of the pathogen have also emerged from the fungal molecular systematic. There is strong molecular evidence that *P. brasiliensis* belongs to the Onygenaceae Family (Onygenales Order, Ascomycota), which must have appeared 150 Ma. This fungal group characteristically produces arthroconidia and aleurioconidia, and has the ability to grow in soil enriched with animal substrates, such as keratin or dung. Phylogenetic analysis also indicates that *P. brasiliensis* is closely related to *Lacazia loboi*, a host-dependent and obligatory parasite, to *Blastomyces dermatitidis*, which has been isolated only sporadically from soil in humid places such as riverbanks, and also to *Emmonsia parva*, a neglected soil fungus associated with rodents. Several recent ecological aspects of *P. brasiliensis* probably are derived from its evolutionary past, especially its long coexistence and adaptation to animal hosts, such as armadillos (Xenarthra Order), established since the Paleocene age (65 Ma) in South America, at that time geologically separate from North America. Better comprehension of the existing biological links of the nine-banded armadillo and *P. brasiliensis* may be helpful to clarify the natural history of PCM, as well as to predict trends in the disease.

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PARACOCCIDIOIDOMYCOSIS: EVOLUTION AND STRAIN TYPING

McEwen, J.G.[1,2]; Montes, B.A.[1]; Garcia, E.A.[1]; Bagagli, E[3].; Restrepo, A.[1]; Taylor, J.W.[4]

[1]Molecular Biology Unit. Corporación para Investigaciones Biológicas. Medellín-Colombia.
[2]Department of Microbiology and Parasitology, School of Medicine, University of Antioquia. Medellín Colombia.
[3]Department of Microbiology and Immunology, Institute of Biosciences, Universidade Estadual Paulista (UNESP), Botucatu, São Paulo, Brazil.
[4]Department of Plant and Microbial Biology, University of California, Berkeley, USA.
E-mail: mcewen@epm.net.co

Introduction: Little is known about the evolutionary biology of the fungus *P. brasiliensis*. However, recent studies have demonstrated that DNA variation correlates with geographical origins of isolates. Whether or not these variations are the product of allopatric speciation remains unknown. Strong evidence exists concerning correlation between genotypic difference (RADP patterns) and fungal virulence. This evidence could well indicate that *P. brasiliensis* encompasses genetically isolated cryptic species. By means of phylogenetic techniques, we are approaching the evolutionary relationships that may exist among *P. brasiliensis* isolates coming from different regions from South America.

Materials and Methods: Sources: Forty two isolates from South America have been collected, Of these, 14 environmental and 10 clinical isolates have been analyzed. Cultures: The isolates were kept and maintained by periodic sub-culturing on BHI agar enriched with glucose. DNA extraction: DNA was prepared from *P. brasiliensis* isolates in their yeast phase. Polymorphic loci: the procedure involved a.) selecting target genes, b) designing PCR primers, c.) amplifying genes from a random sample of the isolates, d.) sequencing representatives of SSCP alleles to find the variable nucleotides. Phylogenetic analysis: DNA sequences from individuals representing unique genotypes were aligned and the variable nucleotide positions were saved. Parsimony trees were then made for each gene using PAUP (PAUP version 4.0.0d62).

Preliminary Results: Phylogenetic analyses were done from the information obtained from the exon2 of the GP43 gene. Partial sequences of the genes corresponding to glucan synthase, chitin synthase, ITS, promotor GP43 and P27 also have been evaluated. These genes, however, showed low polymorphic variability. Using the exon2 of the GP43 gene, a phylogenetic analysis permitted construction of a tree that grouped the isolates by geographic region with strong bootstrap support. This result supports an allopatric speciation process in *P. brasiliensis*, however it is necessary to study more samples and other genes to support this conclusion. We presently are searching for more DNA variation in order to make a more robust phylogenetic analysis in *P. brasilisiensis.* Considering the wealth of the information from the single nucleotide polymorphism, we analyzed the results from the exon2 GP43 and chitin synthase. The chromatographs were evaluated with Phred and Phrap; then the sequences were examined for the presence of polymorphisms using the Sequencher program (Gene Codes Corporation Ann Arbor, Michigan 48108). The genotype corresponding to each isolate was recorded in excel and the haplotype frequencies were calculated using the Arlequin program V2.000. From the 24 isolates that have been haplotyped, 13 variable nucleotide positions have been detected in the exon 2 of the Gp43 gene and arranged in six haplotypes. The chitin synthase intron presented three variable nucleotide positions, arranged in four haplotypes. When the haplotype of both genes were used to identify the environmental isolates from Brazil, four of them shared the same DNA T3B6, T4B14, T7F6, T8B1. A similar observation was made in the Colombian clinical isolates where 4 isolates (P141, P196, P202, P163) shared the same genome.

CHROMOSOMAL POLYMORPHISM AND GENETIC LINKAGE GROUPS OF Paracoccidioides brasiliensis

Cisalpino, P.S.

Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, MG, Brazil.
E-mail: pscisalp@mono.icb.ufmg.br

Paracoccidioides brasiliensis (anamorph; teleomorphic stage unknown), a thermo dimorphic fungus, is the ethiological agent of paracoccidiodomycosis (PCM), a human granulomatous disease prevalent in Latin America. Genetic composition and genomic organization of *P. brasiliensis* is poorly understood. We worked on the electrophoretic karyotype (PFGE) of twelve *P. brasiliensis* isolates, from different geographic areas obtained from

patients with chronic and acute PCM, armadillo and soil. Stable and reproducible karyotypes were observed. Our results were consistent with a haploid number of 4-5 chromosomal bands in the range of 2.0 to 10.0 Mpb. The haploid genome was estimated to be 24-30 Mbp. Densitometric analysis was used to define the size and to characterize co-migration of chromosomal bands. Six distinct karyotype profiles were observed and chromosomal polymorphism made it difficult to correlate banding pattern among isolates. In order to determine chromosome identity, nine specific gene probes have been used to hybridize Southern blots containing intact chromosomal bands. By assignment of homologous gene probes we determined eight different karyotype profiles. The chromosomal polymorphism was impressive, although specific gene probes generally mapped to chromosomal bands of the same size. Three syntenic groups of genes were maintained in the majority of the isolates. The linkage groups are generally conserved suggesting that in spite of gross differences, there is an underlying similarity in the genome organization of different isolates. No correlation could be established between the karyotype profile and the clinical-epidemiological characteristics of the isolates. To study the fungus ploidy, the DNA content of DAPI-stained nuclei was calculated by confocal microscopy and compared to the genome sizes estimated by PFGE. As the yeast phase is multinucleated we also determined the number of nuclei of each isolate. Data were not conclusive on the question of ploidy of *P. brasiliensis*, otherwise indicated the existence of haploid, diploid, and even aneuploid isolates. Even though *P. brasiliensis* is asexual and does not go through a meiotic cycle, we suggest that the chromosomal rearrangements could provide a means for genetic variation in this organism. Mapping of many other genes and probes will be necessary to construct relevant physical linkage groups for the *P. brasiliensis* genome, and to identify genetic markers of interest which may be linked to particular phenotypic traits in this organism. Genetic and physical maps of the *P. brasiliensis* genome would also facilitate the identification and characterization of genetic loci. Having large chromosome segments that span these loci cloned in cosmids, BAC or YACs will help to identify genes involved in the parasite's survival, pathogenicity and diagnosis.

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GENETIC DIVERSITY IN Paracoccidioides brasiliensis AND PATOGENICITY

Puccia, R.

Universidade Federal de São Paulo, São Paulo, Brazil.
E-mail: rosana@ecb.epm.br

The main diagnostic antigen from *Paracoccidioides brasiliensis* is the extracellular gp43 glycoprotein, which is also a protective T-cell antigen in mice and a laminin-binding putative virulence factor. The gp43 purified from supernatant fluids of fungal cultures is a mixture of molecules with about three different isoelectric points of generally neutral values, which can vary among isolates. Previous reports showed that one isolate expressed basic gp43, which was also less antigenic for sera from patients with acute paracoccidiodomycosis (PCM).

Our group has been investigating the diversity of the gp43 antigen at the gene level. Analysis of the polymorphism in the *PbGP43* coding and 5′ non-translated regions from 17 *P. brasiliensis* isolates generated sequence phylogenetic trees that grouped the fungal samples similarly. The three most distant *PbGP43* sequences were translated into basic proteins and were all from isolates of patients with pulmonary PCM. When tested in mice, these isolates produced fewer colony forming units in the spleen during the early stages (21 days) after intraperitoneal infection. Pathogenicity of isolates with different *PbGP43* genetic groups will be further investigated.

Polymorphism in the *PbGP43* is probably reflecting some broader genetic variability in the *P. brasiliensis* isolates, as suggested by Southern blot analysis of total *P. brasiliensis* DNA hybridized with different gene probes. However, a phylogenetic tree based on the polymorphic sites in the ITS1 and ITS2 regions of rDNA showed distinct isolate distribution. It is of note that polymorphism in these regions was not abundant, though.

The *PbGP43* transcription start and end points in several isolates were mapped respectively by primer extension and 3′ RACE. Three start points were found in all four isolates analyzed, but one at –25 from the translation initiation ATG was preferential. Polymorphism in 306 bp of the 5′non-translated region varied from 0 – 4 informative sites. The 3′ region of the *PbGP43* transcript was analyzed in 56 sequences from 10 *P. brasiliensis* isolates. Although the region from the stop codon to the cleavage site was conserved, 13 different poliA cleavage sites were found. The significance of such variability is presently unknown.

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HYGROMICIN B ACQUIRED PHENOTYPE IN Paracoccidioides brasiliensis VIA PLASMID DNA INTEGRATION

Soares, R.B.A.*; Velho, T.A.F.**; Moraes, L.M.P.**; Azevedo, M.O.**; Soares, C.M.A.*; Felipe, M.S.S.**

*Laboratório de Biologia Molecular. ICB, Campus II, UFG, Goiânia, Go, Brasil, 74001-970. **Departamento de Biologia Celular, IB, Campus Darcy Ribeiro, Universidade de Brasília, Brasília, DF, 70910-900, Brazil.
E-mail: mariscel@unb.br.

P. brasiliensis has been studied under the point of view of its biology and physiology by distinct research groups in South America. Along the decades it turned to be an adequate model to study fungal dimorphism and cell differentiation among pathogenic microrganims. Clinical aspects of the infection had also contributed for a better understanding of the paracoccidioidomycosis (PCM). More recently, approaches on molecular biology and new strategies have added a great improvement on the studies related to fungal/host interactions which may be a key to achieve the future for control of this spread disease.

In our opinion, the possibility to manipulate *P. brasiliensis* genome would open the door for specific interactions at molecular level, such as the construction of functional mutants or the improvement of genetic analysis.

In this report we describe a reproducible procedure for the transformation of yeast cells of the human pathogenic fungus *Paracoccidioides brasiliensis* by electroporation with the plasmid pAN7.1 (Punt *et al.*, 1987) harboring the *hph* gene, which confers hygromycin resistance to the transformed cells. We chose the electroporation methodology for its facility on fungal manipulation and cell recovery after a damage. This transformation protocol provides a means for the genetic manipulation of this fungus towards a better understanding of its biology and the mechanisms involved in gene functions, cell differentiation, host-pathogen interactions including virulence and pathogenicity.

Transformation was achieved using electroporation protocol, with intact or linearized plasmid DNA. The fungus was transformed using 200 mM manitol, 5 and 7 KV/cm field strength, 25 μF of capacitance, 400Ω of resistance, 5 μg of plasmid DNA and 10^7 eight days old yeast cells in 400 μL, and selected in overlaid 30 μg/mL hygromycin B (hygB) containing BHI medium. The transformation efficiency obtained was 8-transformants/μg of DNA after the first selection in BHI containing 30 μg/mL hygB. Based on the final hygB resistance phenotype, putative transformants could be divided into two groups. The majority exhibited a high degree of instability losing the resistance phenotype after four passages on selective medium. The other group presented low rate of growth in selective medium. The degree of instability observed was probably due to nuclei number or genomic rearrangements of integrated copies of *hph* gene of *P. brasiliensis* yeast cells. Using hph1 and hph2 primers, it was possible to amplify an expected PCR product of 462 bp in length. All the selected clones, independent of the vector, presented the expected fragment demonstrating the presence of the *hph* sequence into the transformant genomes. Southern blotting analysis also demonstrated the transformation of Pb01 yeast cells. These data certainly represent a great advance on the molecular biology of *P. brasiliensis* since it opens the possibility to direct experiments to study *P. brasiliensis* gene functions involved in host-pathogen interactions, pathogenicity, virulence and/or multi-drug resistance, making possible the development of more specific diagnosis and new approaches to PCM treatment.

Round table 4

TREATMENT OF PARACOCCIDIOIDOMYCOSIS WITH ITRACONAZOLE

Marques, S.A.

Departamento de Dermatologia e Radioterapia. Botucatu-SP, Brazil.
Faculdade de Medicina de Botucatu-Unesp
E-mail: smarques@fmb.unesp.br

The fate of patients with paracoccidioidomycosis (PCM) started to change in 1940 (Ribeiro), with the introduction of sulfapyridine in the treatment. Since then, many other drugs have been used with variable efficacy and different side effects. Introduced in Brazil by Lacaz & Sampaio in 1958, amphotericin B was initially considered as the solution for the disease. However, the side effects like nephrotoxicity, hospital costs, and even the percentage of relapse after treatment (Dillon *et al.* 1986), brought back the chance for new sulfonamide-related drugs and, in the

80', the azoles derivatives. Restrepo *et al.* (1980) and Cucé *et al.* (1981) were the first researchers to show good results with Ketoconazole and to help define the best regimen of dose and time of treatment. Despite the efficacy of ketoconazole, the potential severity of some side effects challenged the pharmaceutical companies to pursue safer azoles derivatives drugs. Itraconazole was one of the so called "second generation azoles antifungals compounds". The first published trials using itraconazole appeared in 1987 (Negroni et al., Restrepo et al. and Borelli). The initial and subsequent results were very auspicious. However, the large range of drug interaction of itraconazole, by inhibition of the CYP 3A4 enzyme of cytochrome P-450 system, has been a matter of concern. In addition, its costs and evidences of therapeutic failure in severe cases have been debated as to the real importance of itraconazole among other effective drugs against *Paracoccidioides brasiliensis*. The following experience started in 1989 and its objective was to identify percentage of relapses after extended follow-up of patients with paracoccidioidomycosis treated with itraconazole. Although we have observed that 75% of relapses after amphotericin B occurred in the first three years after treatment, the extended follow-up, at least two years, was chosen and accepted as the best parameter of efficacy in terms of therapeutic analysis. In short, 58 patients, median age of 44 years, were classified in terms of clinical forms and severity index following Franco et al. and Mendes' proposals respectively. Twenty-four patients with mild or moderate clinical conditions received 100mg/day of itraconazole for six months and 34 patients with moderate to severe and severe clinical conditions were treated with 200 mg/day in the first month and 100 mg/day for another six months. From two to nine years after the treatment, 34.5% of the patients discontinued the follow-up (27.6%) or died of unrelated causes (6.9%). Among the remaining 38 patients, there was one failure (2.6%) and 8 (21.0%) relapses. Three patients relapsed from two to three years after treatment, four from three to five years and one seven years after the treatment. In conclusion, although these data are better than those presented by Dillon (1973) as to extended follow - up after using amphotericin B, it is very important to pursue better therapeutic procedure for patients under itraconazol, and to consider maintaining therapeutic regimen with sulfonamides for the severe cases.

PARACOCCIDOIDOMYCOSIS AND THE INFLUENCE OF ITRACONAZOLE TREATMENT ON LUNG ABNORMALITIES: EXTENDED FOLLOW-UP OBSERVATIONS

Tobón, A.M.*; Agudelo, C.A.; *Osorio, M.L.† and Alvarez, D.L.‡ and *Restrepo, A.

*Grupo de Micología Médica y Experimental, Corporación para Investigaciones Biológicas (CIB), Medellín, Colombia. †Hospital La María,‡Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia.

Paracoccidioidomycosis (PCM) produces lesions in various organs and systems but mainly in the lung as shown by its involvement in 80% of the active cases and by the presence of residual fibrosis in 60% of the patients. Among the treatments presently in use itraconazole (ITZ) has shown high cure rates (over 95%) and low relapse figures (3%). Despite the adequate control exerted by therapy on active disease, the fibrotic sequelae appear not to be influenced by such treatment thus hindering the complete restoration of the patient's health.

Tò better define this problem we analyzed the records of 52 PCM patients treated with ITZ who have had prolonged (over a year) post-therapy follow-ups and lung X-ray films. They were adult males, 84.5% with the chronic multifocal disease and 15.5% with the acute-subacute, disorder. The patients received 100-200 mg ITZ per day for variable periods according to the severity of the mycosis. Most (70%) patients with chronic PCM were treated for 3-6 months while those with juvenile-type disease (30%) had a 6-12 months ITZ course. The mean follow-up period was 7,5 years distributed as follows: 1-5 years: 28, 6-10 years: 15, and 11-17: 9 patients. Parenchymal involvement was classified according to extension of infiltrates and fibrosis following the international standards for pneumoconiosis, which divide the lung in 6 fields. If 1-2 fields had lesions involvement was minor, if 3-4, moderate and if 5 or more, severe. Various statistical analyses were used to determine significance of the findings.

At diagnosis, 45 (85.6%) patients exhibited respiratory symptoms represented by cough (75%), expectoration (63.4%), and dyspnea (55.8%). Extra-pulmonary manifestations, as well as fever and weight loss, were present in over 50% of the patients. At the end of the post-therapy study, most symptoms had disappeared with the exception of respiratory complaints persisting in half of the cases. The latter manifestations resolved or appeared *di novo* in some patients for a total of 24 (46.1%) patients with these symptoms at the end of the observation period.

The initial lung X-ray films revealed infiltrates in 44 cases (84.6%) while 8 patients (15.3%) had no lung abnormalities. Infiltrates were mainly interstitial (59.6%), followed by mixed alveolar plus interstitial infiltrates (29.5%). At the end of the observations, interstitial infiltrates were still present in 13 (41.9%) of the patients with this pathology at diagnosis; mixed infiltrates were observed in 4 (12.9%), resulting in total of 17 (38.6%) with residual problems. At diagnosis, the severity of the infiltrates was minor in 8 patients, moderate in 18 and severe in another

18. At the end, infiltrates' intensity had either decreased in 60% of the patients or remained unchanged in the remaining cases.

As for fibrosis, this abnormality was absent in 38 (73.0%) patients at time of diagnosis; the remaining 14 (26.9%) already exhibited this residual lesion; from the latter, 10 had minor, 3 moderate and 1 severe damage. Post-treatment, no patient cleared on the contrary, 11 had developed fibrosis *di novo* for a total of 25 (48.0%) patients with the sequelae. Increased damage was recorded in 4 patients (28.5%) while in the remaining no changes in fibrosis' intensity were noticed. Bullas were noticed in 12 patients at diagnosis and in 26 (50%) at that end. No significant differences were noticed when comparing fibrosis to time of follow-up.

Attempts were made to predict the development of fibrosis on the basis of the intensity of infiltrates at diagnosis and of fibrosis at the end. From 16 patients who at diagnosis had no infiltrates or had minor intensity involvement, only 1 developed fibrosis (6%). Of 18 patients with infiltrates of moderate intensity (3-4 fields) when first diagnosed, 50% showed fibrotic sequelae at the end. Of 18 patients with infiltrates involving 5 or more lung fields, 83% developed fibrosis. Consequently, it appears that the degree of involvement at diagnosis marks the outcome of the residual sequelae. If active lesions could be more promptly intervened by treatment, the prognosis of PCM patients could be improved.

TREATMENT OF PATIENTS WITH PARACOCCIDIOIDOMYCOSIS: NEW INSIGHTS AND APPROACHES

Telles, F.Q.

Head of the Laboratory of Mycology, Dept of Infectious Diseases, Federal University School of Medicine.
R. Capitão Souza Franco, 520, apto. 7, cep:80730-420, Curitiba, PR, Brazil.
E-mail: queiroz.telles@netpar.com.br

Differently from other pathogenic fungi, *Paracoccidioides brasiliensis* is a very sensitive organism when exposed to antifungal drugs. Even the sulfonamides, can promote its growth inhibition. According to its sensibility profile, a large therapeutic armamentarium for patients with paracoccidioidomycosys is available. Several classes of antifungal drugs were employed by many authors in the treatment of paracoccidioidomycosis, including the sulfonamides (sulfadiazine, sulfadoxine, sulfamethoxypyridazine, cotrimazine and cotrimoxazole), amphotericin B, the azole compounds (ketoconazole, fluconazole and itraconazole) and terbinafine. The cure rates achieved with these various drugs have ranged between 69% and 100%.

Although comparative clinical trials in paracoccidioidomycosis are lacking, it is believed nowadays that itraconazole is the drug of choice for most clinical forms of the disease. In order to compare the clinical efficacy of cotrimoxazole and itraconazole in paracoccidioidomycosis, we evaluated a cohort of 217 patients treated at the Hospital de Clínicas, Universidade Federal do Paraná in Brazil. One hundred and fifty nine patients were treated with cotrimoxazole (160 mg of trimethoprim/800 mg of sulphametoxazole TID for 30 days followed by the same dosage BID) were compared to 58 patients who received itraconazole (100 mg BID for 3 months followed by 100 mg daily). The response was accessed by clinical, radiological and serological criteria. Thirty patients in the itraconazole arm were previously treated with cotrimoxazole and/or amphotericin B. The groups were comparable in terms of age, gender and clinical form (acute or chronic). Pulmonary and lymph node involvement were more frequent in patients receiving itraconazole ($p=0.009$ and $p=0.006$ respectively). The initial serum antibody titer was higher in patients receiving itraconazole. The cure rate was 62% for patients receiving cotrimoxazole and 81% for the group receiving itraconazole. The median duration of treatment was 7 (6-12) months in the group receiving itraconazole and 24 (15-42) months in the group receiving cotrimoxazole ($p<0.0001$). Forty-five patients (28%) in the group receiving cotrimoxazole abandoned the treatment compared to 08 (14%) in the group receiving itraconazole ($p=0.03$). Post treatment follow up was achieved in 159 and 58 patients from the cotrimoxazole and the itraconazole groups respectively. Seven relapses were observed in the cotrimoxazole group versus 01 in the itraconazole group, after 06 to 24 months after the therapy.

Among the new antifungal compounds, the cell wall inhibitors (caspofungin and micafungin) do not show *in vitro* action against *Paracoccidioides brasiliensis* and they were not evaluated in paracoccidioidomycosis. On the other hand, the new itraconazole formulations (oral solution and intravenous solution) may be helpful therapeutic tools, especially for those patients presenting itraconazole low plasma levels, due to the capsule formulation pharmacokitetic profile. Voriconazole a new triazole obtained form the modification of fluconazole molecule, presenting both oral and intravenous formulations, will be also a member of the paracoccidioidomycosis therapeutic armamentarium. In a prospective controlled clinical trial, voriconazole is been evaluated in a multicentric study to verify its efficacy and safe in patients with paracoccidioidomycosis. Preliminary results obtained in 42 patients, show

that voriconazole at the daily dose of 400 mg, can achieve clinical, radiological and serological responses after the mean time of 6 months therapy. Voriconazole has also a potential use in neuroparacoccidioidomycosis due to its good central nervous system penetration.

According to the presented data, we can conclude that most of the patients with paracoccidioidomycosis can be treated with short courses of itraconazole or voriconazole (mean of 06 months). The post-azolic sulfonamide maintenance treatment is not needed. Cotrimoxazole can be successfully used during 15 months in the mild to moderate clinical forms of paracoccidioidomycosis. Patients needing intravenous antifungal drugs can receive intravenous cotrimoxazole, itraconazole, voriconazole or amphotericin B.

Beside the antifungal therapy, the general measures should never be forgotten during the management of paracoccidioidomycosis. These actions include the control of tabagism and or alcohol intake, suitable diet, treatment and control of co-infections like tuberculosis, enteroparasitosis and bacterial infections.

TREATMENT OF PARACOCCIDIOIDOMYCOSIS PATIENTS: DURATION OF TREATMENT, MAINTENANCE THERAPY, RELAPSES AND SEQUELS

Shikanai-Yasuda, M.A.; Yoshida, M.; Freitas, V.L.T.; Bernard, G. and Ferrari, V.

Ambulatório de Micoses Sistêmicas e Clínica de Moléstias Infecciosas e Parasitárias
Laboratório Imunologia Médica HC-FMUSP
Hospital das Clínicas Faculdade de Medicina USP (HC-FMUSP), São Paulo, SP, Brazil.
E-mail: dmip.secr@hcnet.usp.br

Paracoccidioidomycosis was diagnosed through mycological, serological or histopathological methods in 38 patients prescribed to receive 50-400 mg of itraconazole daily from three to thirteen months. Eleven patients were excluded from this group for further analysis because of shorter periods of treatment or after having received simultaneously or previously other antifungal drugs.

Most of patients were male (81.5%); mean age was 44.2 +/- 4.8 years old and 25.9% presented with the acute form of the disease, 66.7% with multifocal chronic form, 7.4% with unifocal chronic form. One 28-years old HIV coinfected patient had cervical lymph node enlargement and has been prescribed 400 mg daily of itraconazole for the last 8.5 months. Mean duration of treatment was 66 +/- 3.0 months, for the acute form it was 6.7 +/- 3.2 months and the chronic form, 6.8 +/- 2.9 months. Marked clinical improvement was noticed in all cases, expressed by for at least 90% decreasing in the number or intensity of the lesions.

As maintenance therapy, sulfametoxipiridazine was employed in 23 out of 25 patients until negativativity or stabilization in low levels of antibody serological tests. No relapses were noticed except for two patients without regular maintenance therapy: one patient with splenomegaly and laryngeal envolvement received six month of 100 mg daily of itraconazole with improvement and after interruption of maintenance therapy (slow sulfa), presented lymph node enlargement and hepatoesplenomegaly. Another patient with acute form received 400 mg daily of itraconazole for six months and was left without maintenance therapy. He returned 45 days later with lymph node enlargement and had been prescribed again 400 mg daily of itraconazole with improvement and no relapse after.

In this studied group three patients were submitted to tracheostomy and laryngeal stenosis was diagnosed during the follow up period. In two patients, laryngeal sequel was observed. Chronic obstructive pulmonary disease was also observed in two other patients.

Fourteen of this 29 patients had been included in a randomized study (Med. Mycology in press) and received for 4 to 6 months 50-100 mg of itraconazole daily from 1988 to 1992, in comparison with 14 patients treated with ketoconazole 200 mg daily and sulfadiazine 150 mg/kg/day up to 6 g daily. All patients in itraconazole and sulfadiazine groups and 13 in the ketocotiazole group showed a good clinical response to the chemotherapy. One patient in the ketoconazole group showed therapeutic failure according to clinical and mycological criteria. No significant difference was observed among these groups concerning clinical parameters or decrease on serological titers up to ten months of treatment, by contraimmunoelectrophoresis. Untoward effects of chemotherapy, though present, were not severe enough to require discontinuation of treatment in these patients.

Considering higher doses of itraconazole (up to 200 mg daily), we emphasize the need to extend itraconazole treatment by at least 12 months in cases similar to the acute cases mentioned above. Additionally, incidence of Central Nervous System lesions (more than or equal to 20% according to our unpublished results), needs to be considered to select the better drug for the treatment of active systemic disease. Finally, in order to find the best duration of treatment, it is important to establish reliable narkers for follow up control in prospective studies.

Round table 5

Paracoccidioides brasiliensis 87 KDA ANTIGEN, A HEAT SHOCK PROTEIN USEFUL IN DIAGNOSIS OF PARACOCCIDIOIDOMYCOSIS

Díez, S.[1,2*]; Gómez, B.L.[1,2], Restrepo, A.[2]; Hay, R.J.[1] and Hamilton, A.J.[1]

[1]St. John's Institute of Dermatology, Guy's Hospital, London, England.
[2]Corporación para Investigaciones Biológicas, Medellín, Colombia.

The 87 kDa antigen derived from the fungal pathogen *Paracoccidioides brasiliensis* can be detected in the sera of infected patients and its levels have been shown to correlate well with response to treatment and clinical cure. Despite its potential importance the antigen has been poorly characterized. The 87 kDa antigen was purified to homogeneity via preparative gel electrophoresis; N-terminal amino acid sequencing revealed substantial homology with heat shock proteins (hsps) from a variety of organisms. A monoclonal antibody (MAb) raised against a *Histoplasma capsulatum* 80 kDa hsp showed cross reactivity to the purified 87kDa antigen via Western blot, and the 87 kDa specific MAb P1B demonstrated that the antigen was expressed at higher levels in yeast compared to mycelia using the same technique. ELISA and immunofluorescence reactivity using P1B confirmed increased expression of the 87 kDa antigen during the temperature-induced transformation of mycelia to yeast. Yeast to mycelial transformation was accompanied by a fall in expression, although the 87kDa antigen was clearly constitutively expressed in both phases. An ELISA for the detection of antibody response against the purified 87 kDa-hsp protein has been developed; the results indicate that it is possible to employ this protein for the immunological diagnosis of PCM. Immunochemical staining of tissues with MAb P1B from patients infected with *P. brasiliensis* confirmed in vivo expression of the 87 kDa antigen by yeasts, and identification of this antigen via this method appears to be a useful adjunct to other methods used to diagnose paracoccidioidomycosis (PCM).

THE RECOMBINANT Histoplasma capsulatum ANTIGENS IN DIAGNOSIS OF HISTOPLASMOSIS

Zancopé-Oliveira, R.M.

Serviço de Micologia do Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil.
E-mail: zancope@cpqhec.fiocruz.br

Histoplasmosis is a systemic fungal disease caused by *Histoplasma capsulatum* variety *capsulatum*, a dimorphic fungus that grows in the mycelial form at room temperature and in the yeast form at 37°C or in infected tissues. The diagnosis of histoplasmosis is based on the results of clinical evaluation and associated laboratory tests. Isolation of *H. capsulatum* provides a definitive diagnosis, but it is time-consuming and lacking in sensitivity. In such cases, serologic evidence of this mycosis is an important contribution. The H and M antigens of H. capsulatum are glycoproteins that form the basis for the specific serological diagnosis of histoplasmosis. Diverse immunoassays have been used to detect specific antibodies and fungal antigens. Meanwhile, 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. Nucleic acid-based technologies have been progressively adapted in the diagnosis of histoplasmosis. We shall review the cloning and characterization of the genes encoding H and M antigens, and the identification of predicted B-cell epitopes from both proteins. Development of serological tests that utilize the recombinant fusion H and M proteins and a PCR reaction as an alternative method for identification of H. capsulatum will also be discussed.

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CIRCULATING ANTIGENS IN PARACOCCIDIODOMYCOSIS (gp43) AND SOME ECO-EPIDEMIOLOGICAL ASPECTS

Camargo, Z.P.[1]; Silva, S.H.M.[1]; Ono, M.A.[2]

[1]Universidade Federal de São Paulo (UNIFESP) and[2]Universidade Estadual de Londrina, PR, Brazil.
E-mail: zoilo@ecb.epm.br

Circulating antigens. The precise diagnosis of paracoccidioidomycosis (PCM), in most cases, is established by direct methods and indirect serological tests. The latter method is reliant on the identification of the host's humoral responses, which are usually impaired or absent in patients with severe forms of PCM and also in patients with decreased cellular response. The diagnosis and activity of the disease are measured on the level of specific antibodies, however, sometimes they remain elevated or persist stationary. So, alternative tests are needed to diagnose the disease, such as antigen detection techniques. In this study we describe the development of an inhibition ELISA for detection of circulating antigen with monoclonal antibodies anti-*P. brasiliensis'* gp43. A total of 57 patients with mycologically confirmed and active PCM, fluid cerebrospinal (FCS)from other 14 patients with neuroparacoccidioidomycosis, and broncoalveolar lavage (BAL) from 13 other PCM patients were used in this study. Human health sera from the bank of blood donors were used as negative controls. Our results showed that overall, 94.7% of the 57 PCM serum samples had detectable levels of circulating gp43 antigen above the cut off point with a mean antigen concentration of 7.75 microgram/ml. Circulating gp43 antigen was detectable in all patients with the acute form of PCM (mean, 5.3 microgram/ml), and 80.43% of those patients with the chronic form (mean, 8.32 microgram/ml). No cross-reactions were observed in heterologous serum samples, specifically with histoplasmosis, criptococcosis sera. Inb-ELISA was able to detect gp43 in 100% of CRL and BAL, with mean antigen concentration of 17.4 microgram/ml and 16.6 microgram/ml, respectively. Inb-ELISA showed useful to detect gp43 in biological fluids.

Eco-epidemiology. We evaluate the effect of ten pesticides on *P. brasiliensis* and associated these data with the difficulty in isolating the fungus from agricultural soil. Six fungicides (Alto 100. Benlate, Captan, Dithane, Plantacol, Rovral), two herbicides (Pivot, Roundup) and two insecticides (Azodrin, Curacron) were evaluated. All pesticides assayed inhibited *P. brasiliensis* in a dose dependent manner, and a great variability among ED50 was observed. The inhibitory effect of pesticides on *P. brasiliensis* suggests that they can interfere with attempts to isolate it from soil, where tones of pesticides are applied at large scale in several crops.

Sera from 305 dogs were analyzed by ELISA to determine the presence of specific anti-gp43 antibodies. The dogs were divided into three groups according to the origin: urban dogs (animals with little or no contact with rural areas), suburban dogs (from the urban outskirts) and rural dogs. There was a significant difference between groups. Rural dogs reacted positively in 89.5% cases, followed by suburban (48.8%) and urban dogs (14.8%). On the other hand, dogs were skin tested with gp43, and suburban and rural dogs showed positivity of 13.1 and 38.1%, respectively. These results indicate that ELISA and skin testing can be useful in the epidemiological study of PCM in dogs and that encounter with the fungus in nature is a frequent event.

We also evaluated the susceptibility of dogs to develop PCM by experimental infection. Puppies were inoculated with *P. brasiliensis* by an intravenous route and 2 out 4 died one week post inoculation, showing at histopathological analysis, granulomas in the lungs, spleen and liver. *P. brasiliensis* was isolated from these organs. The animals that survived the infection showed a strong reaction when skin tested with gp43. These animals were sacrified at one and five month after infection, and no lesions, macroscopic or microscopic, were observed in the lungs, spleen, and liver, furthermore no *P. brasiliensis* culture was obtained from these organs. These results suggest that dogs can develop PCM and reinforces the importance of this animal as sensitive indicators of *P. brasiliensis* in the environment.

EPIDEMIOLOGY AND SEROLOGICAL TESTS IN DIAGNOSIS AND PATIENT FOLLOW-UP OF PARACOCCIDIOIDOMYCOSIS

*Mendes-Giannini, M.J.S.; *Salina, M.A.; *Monteiro da Silva, J.L. and †Mendes, R.P.

*Faculdade de Ciências Farmacêuticas de Araraquara – UNESP – SP - Brazil, †Faculdade de Medicina de Botucatu – UNESP – SP - Brazil.
E-mail: giannini@fcfar.unesp.br

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by the dimorphic fungus *Paracoccidioides brasiliensis*, which often affect rural workers, usually male adults. The incidence of the disease is not homogeneous within the endemic area, and it has also become an important endemic disease in some new regions. The overt disease is manifested by two clinical forms, acute/subacute and chronic. Serological methods are of considerable value in PCM and various tests have been employed in the diagnosis and monitoring of the patients response to treatment; they rely largely on antibody detection and some of them use purified or recombinant antigens but the majority of laboratories employ crude extracts and low sensitivity tests. Even so, we have some problems with the technician training. Furthermore, several studies have shown that the detection of antigens instead of antibodies is a powerful tool for diagnosis, monitoring of treatment and cure control. Particularly, for early diagnosis, or in cases of immunocompromised individuals or when antibody detection is non-conclusive. In addition, the monitoring of treatment response by measuring antigen levels would be an asset, since antibody titer may remain elevated even after apparent clinical remission. Antigenemia and antigenuria correlate well with improvement or worsening of the disease. The detection of antigen may be a test that would permit a more accurate characterization of cure in PCM patients. Thus, serological tests for antibodies and antigens, should be considered together with epidemiology date and clinical state of patients.

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Round table 6

DYSFUNCTION OF THE IL-12/IFN-γ AXIS IN PARACOCCIDIOIDOMYCOSIS PATIENTS

Romano, C.C.

Fellow, Laboratory of Clinical and Experimental Allergy and Immunology, Faculdade de Medicina, USP, Av. Dr. Arnaldo, 455, sala 2345, São Paulo, cep 01246-903, SP, Brazil.
E-mail: romanocc@hotmail.com.br

Our previous results with *in vitro* stimulation with the 43 kDa glycoprotein from *P. brasiliensis* of peripheral blood mononuclear cells (PBMC) of patients with PCM demonstrated a predominant antigenic-specific Th-1 immunosuppression characterized by low or absent production of the cytokines IL-2 and IFN- gamma which was associated with high levels of anti-gp43 antibodies and significant production of the down-modulating cytokine IL-10. These findings suggest the presence of an imbalance in the production of IL-12 and IL-10, which have opposite effects on the immune response by directing to Th-1 and Th-2 type responses respectively. The Th-1 type response, identified by the dominant participation of IL-2, IL-12 and IFN- gamma has been associated with resistance to several infectious diseases like tuberculosis, hanseniasis and coccidioidomicosis, whereas the Th-2 type response, identified by the dominant participatin of IL-4, IL-5, IL-10 and IL-13 is associated with susceptibility. The *in vitro* modulation of the immune response of the patients with IL-12 resulted in an increase in IFN-gamma production, although it was not able to completely revert in every patient the down-modulation of the Th-1 response, possible because it could not overcome the inhibitory influence of IL-10. This was corroborated by experiments showing that (a) addition of a neutralizing anti-IL-10 antibody increased the IFN-gamma production to gp43 the concurrent, (b) the concurrent addition of anti-IL-10 and IL-12 not only increased further the IFN- gamma production but also restored the lymphoproliferation in response to gp43 of the patients.

Based in these findings, we then proposed to investigate the production of IL-12 in the supernatants of cultures of PBMC from patients with active PCM in presence of gp43 and a control fungal antigen, from *Candida albicans* (CMA). As expected, we found reduced levels of the biologically active IL-12p70 in gp43 cultures compared to those with CMA and to those in cured controls' PBMC cultures with gp43. The biological activity of IL-12 is mediated by the expression of its receptor by the lymphocytes. This receptor is constituted of 2 sub-units, one (beta1) constitutively expressed e one (beta2) regulated by other cytokine. The (beta1) sub-unit was equivalently expressed by lymphocytes of both patients and controls. However, the beta2 sub-unit was poorly expressed by patients' lymphocytes compared to the controls. Therefore, the imbalance of the IL-12 and IFN-gamma pathway, associated with the significant levels of IL-10 that are produce by the monocytes early after being challenged by the fungus, help to explain the lack of a Th-1 type immune response and the defect in the cellular immune response of such patients, which are not observed in cured individuals. In conclusion, in PCM, as in other infectious diseases, the dysfunction of the IL-12/IFN-gamma axis, is apparently related to the inability of the host immune system in controlling the fungal dissemination and the consequent development of disease.

HUMAN MONOCYTES IN PARACOCCIDIOIDOMYCOSIS: FUNGICIDAL ACTIVITY AND CYTOKINES PRODUCTION

Soares, A.M.V.C.

Department of Microbiology and Immunology. Instituto de Biociências – UNESP - Botucatu, cep 18618-000, SP, Brazil.
E-mail: acsoares@ibb.unesp.br

Monocytes and macrophages are components of innate immune response that play an important role against systemic fungal infections. Imbalance in supressor or stimulatory cytokines secretion by these cells may determine the development of the disease or microrganism death, and can influence the nature of the adaptative immune response.The aims of our work was to evaluate the production of cytokines by monocytes from patients with active paracoccidioidomycosis, and to analyse the profile of cytokines *in vitro*, using monocytes from healthy subjects challenged with high and low virulent strains of *P. brasilensis*, as well the kinects of the mRNA cytokine expression by RT- PCR after 4,8,12,18 and 48h of infection. The results showed that the endogenous levels of TNF-α, IL-1β, IL-6, IL-8, IL-10, and TGF-β1 detected in monocytes supernatants of patients cells, cultivated without stimulus, were significantly higher than those from healthy controls. However, patients monocytes showed a significantly lower levels of TNF-α and IL-6 in response to LPS. IL-1β, IL-6, IL-8, IL-10 concentration in LPS-stimulated cultures were higher in patients than in controls. These results demonstrated that monocytes from patients with active paracoccidioidomycosis produce both inflammatory and anti- inflammatory cytokines. The impairment in TNF-α and IL-6 synthesis after LPS stimulation suggests that IL-10 and TGF-β1 may play a role in dowregulation of these cytokines production by monocytes. The study of the profile of cytokines showed that both *P. brasiliensis* strains induced the production of IL-1β, IL-6, IL-10 and TNF-α by infected monocytes. However, Pb18 strain induced higher levels of IL-1β, IL-6 and IL-10 than Pb265 strain. IL-8 and TGF-β1 levels were not detected in these cultures. The mRNA cytokine expression showed similar results when compared to supernatant cytokines measured by ELISA.

An other aim of our study was to evaluate the mechanisms by which normal activated monocytes kill *P. brasiliensis in vitro*. The results showed an involvement of H_2O_2 , but not superoxide anion and nitric oxide. Moreover, we demonstrated that human nonprimed monocytes inhibits H_2O_2 release by a mechanism dependent on prostaglandin synthesis. We suggested that this mediator release induced by high virulent strain of *P. brasiliensis* may represent a fungus escape mechanism from the effector function of phagocytic cells. The role of supressor cytokines such as IL-10 and TGF-β on the fungicidal activity of activated human monocytes was also evaluated. IL-10 inhibited the fungicidal activity presented by TNF-α activated cells, showing the capacity of this cytokine to deactivate human monocytes for *P. brasiliensis* killing. On the contrary, TGF-β increased the fungicidal activity presented by either nonactivated or TNF-α activated cells. Our studies also demonstrated that the activation mechanism by cytokines of human monocytes for *P. brasiliensis* killing is highly modulated by prostaglandins. The results demonstrated that when prostaglandins synthesis is inhibited by cells preincubation with indomethacin, low concentrations of TNF-α are sufficient for monocyte activation for *P. brasiliensis* killing. However, in the presence of prostaglandins the cells must be activated by two signals, one given by IFN-γ and other by adequate concentrations of TNF-α. Thus, the fungus killing by phagocytic cells may be dependent on the balance of mediators with pro- and anti- inflammatory activity produced during the contact between *P. brasiliensis* and these cells.

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MODULATION OF THE IMMUNE RESPONSE DURING THE INFECTION WITH Paracoccidioides brasiliensis

Silva*, J.S.; Campanelli*, A.P.; Souto+, J.T; Livonesi*, M.C.; Martins*, G.A.; Barbosa*, A.C.C.; Moreira*, A.P.; Pereira*, M.S.F.; Figueiredo++, F.; Maffei*, C.M.L.; Martinez*, R. and Rossi*, M.A.

*School of Medicine - USP, Ribeirão Preto, SP, +UFRN, Natal, RN, ++UNB, Brasília, DF; Brazil.
E-mail: jsdsilva@fmrp.usp.br

The mechanisms that mediate and regulate the inflammatory and immune response induced by the fungus *Paracoccidioides brasiliensis* (Pb) as well those that determine resistance or susceptibility to the infection are poorly

known. Here, we investigated the role of several cytokines and adhesion molecules in the resistance to Pb infection. We found that mice genetically deficient in IFN-gamma, TNF-alpha receptor p55, or IL-12 are involved in the resistance to Pb infection, in the modulation of granuloma formation, and in the control of fungus dissemination. Mice deficient of CD28, ICAM and iNOS were also more susceptible to infection. On the contrary, the yeast cells growth in IL-4KO was lower than in WT mice. Typical inflammatory granulomas were found only in (wild type) WT mice while the susceptible presented disseminated granulomas. In resistant mice we found widely spread focal collections of lymphomononuclear cells associated with a few number of yeast forms.

As during the infection with Pb there is a constant mobilization of leukocytes and granulomatous inflammatory reaction around the yeast forms, it is reasonably that chemokines are actively involved in the recruitment of leukocytes to inflammatory sites. In fact, we found an association between MIP-2, KC and MIP-1alpha and neutrophils infiltration, in the lungs of WT mice in the early acute phase of infection. Further, we also observed a direct correlation between high levels of RANTES, MCP-1, IP-10 and Mig and mononuclear cell infiltration in tissues from WT mice. However, in lungs from GKO mice, we observed a delayed increase in MIP-2, KC and MIP-1alpha production, which was followed by high neutrophil infiltration. Moreover, chemokine receptor analysis revealed a change in the receptor profiles expressed by WT versus GKO animals. Higher expression of CXCR3 and CCR5, together with lower levels of CCR3 and CCR4, were observed in lungs of WT mice. In GKO mice, on the contrary, higher expression of CCR3 and CCR4 and lower expression of CCR5 and CXCR3 was observed. Consistently, spleen cells from Pb 18-infected WT and GKO mice migrated preferentially in response to IP-10 and eotaxin stimulation, respectively. Together, these results suggest that the chemokines determine the composition the inflammatory cells found in the lesions induced by Pb. Moreover, that the cell types of the lesions determine if the animal will be resistant or susceptible to the infection

We also investigated the mechanisms implied in mediating the observed immunosupression following Pb infection. As described before, only 10% of PBMC of the patients studied showed lymphoproliferative response to PHA. In a sharp correlation, the expression of CTLA-4 and the levels of apoptotic cells were significantly increased in PBMC of all patients compared with that found in cells of normal subjects. Moreover, antigen-induced-CD4 T cells activation increased the levels of apoptotic cells from patients, but not from normal subjects. Additionally, the blockade of Fas/FasL interaction did not restore proliferative response of T cell from PCM patients, but led to a reduction of the apoptotic cells. Interestingly, the concomitant blockage of CTLA-4 and FasL led to an increased proliferative T cell response to PHA, but not to the fungal antigen used. Overall, the data indicated that Fas-FasL-mediated apoptosis and that the stimuli driven by CTLA-4 engagement are implied in modulating the immune response in Pb-infected patients.

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THE MACROPHAGE AND Paracoccidioides brasiliensis CONIDIA: INTERACTIONS MEDIATED BY NITRIC OXIDE.

Luz Elena Cano R. and members of CIB´s Mycology Group.
Medical and Experimental Mycology Group, Corporación para Investigaciones Biológicas, CIB, and Bacteriology and Clinical Laboratory School, Universidad de Antioquia, Medellín, Colombia.
E-mail: luz_elena_cano@hotmail.com

Histological studies have indicated that the dimorphic fungus *Paracoccidioides brasiliensis* (*P. brasiliensis*), the aethiological agent of paracoccidioidomycosis (PCM), is regularly phagocytozed by macrophages (M-theta); furthermore, it has been experimentally shown that if these cells are blocked, dissemination of the infection takes place thus indicating that they restrict fungal multiplication.

Certain interactions between murine M-theta´s and *P. brasiliensis* (yeast or conidia) have been the subject of previous studies. It was reported that gamma interferon (IFN-gamma)-activated macrophages (IFN-gammaM-theta´s) exhibited a potent fungicidal activity against this pathogen suggesting that the fungicidal mechanism was independent of the respiratory burst´s products.

The cytotoxicity of activated M-theta was shown to be dependent of L-arginine pathway. This mechanism, induced by IFN-gamma and other cytokines, is mediated by the synthesis of nitrogen oxidants, specifically nitric oxide (NO), a compound formed endogenously via conversion of L-arginine to citruline, thanks to the action of the inducible nitric oxide synthase (NOS-2 or iNOS). The main NO cytotoxic activity appears to depend on the interaction occurring between the ferrous iron present in the soluble guanilate cyclase hem molecule. The end-results of the NO-combination with iron-sulfur centers present in the enzymes of the mitochondrial respiration system, as well as in the DNA synthesis pathway of target cells, is also of importance.

Recent *in vitro* studies have revealed an important role for NO in the fungicidal mechanism of IFN-γ-M-theta´s against *P. brasiliensis* conidia. It was shown that these M-theta´s when infected with conidia were capable of producing high NO levels and as a result, inhibited their transformation to yeast cells in a significant manner. Both process were shown to correlate inversely; furthermore, the use of NO inhibitors/blockers, such as L.NMMA, arginase and aminoguanidine (AG) reverted both phenomena. Thus, it became clear that NO is involved in the *in vitro* killing of *P. brasiliensis* conidia by IFN-gamma-activated-M-theta´s.

The *in vitro* fungicidal activity was also evidenced through participation of iron in this NO-mediated mechanism. Thus, the addition of iron donors, such as holotransferrine or $FeSO_4$, to IFN-gamma activated M-theta´s, reverted the inhibitory effect observed on the transformation of conidia to yeast .

Additionally, the intranasal inoculation of $4x10^6$ viable *P. brasiliensis* conidia in male BALB/c mice resulted in a chronic progressive pulmonary disease which was characterized histopatologically by granuloma formation. This experimental model of PCM has revealed several findings concerning the host´s pulmonary responses during the development of this infection, as follows: 1) Inflammatory period (24h to 1 week) with high proinflammatory cytokines (piCKS) levels such as TNF-alpha, non significant production of NO and mRNA-iNOS expression and low iNOS+ cells, 2) Transitional period (1 to 4 weeks) characterized by a mixed pattern of CKs, beginning of granuloma formation, presence of elevated levels of IFN-gamma, increase of the mRNA iNOS expression and in the number of iNOS+ cells, and 3) granulomatous period (8 to 16 weeks) marked by intense granuloma formation, high mRNA- IL-10, TNF-alpha and iNOS expression, and the highest numbers of iNOS+ cells, located within the granuloma. It was found that iNOS was preferentially expressed in the granuloma and specifically in the epithelioid histiocytes. When the animals were treated with aminoguanidine (AG), an iNOS inhibitor, a significantly reduced survival time in infected animals but treated with this inhibitor was observed. All these results suggest a protective role for NO during the chronic period of the infectious process.

Nonetheless, other authors have shown that C57BL/6 mice inoculated with *P. brasiliensis* yeast cells produced high NO levels; a production that appeared to be involved in immunosuppression due to the fact that NO also inhibits lymphoproliferation (Bocca *et al.*, 1998). More recently, Nascimento *et al.* (2002) suggested a dual role for NO in PCM: essential in resistance but overproduced when associated to susceptibility.

It appears that is now feasible to attempt defining the role of the NO molecule on cellular and molecular mechanisms participating in the pathogenesis of experimental PCM.

ENHANCED PRODUCTION OF MACROPHAGE INFLAMMATORY PEPTIDE-1alpha BY ALVEOLAR MACROPHAGES FROM PATIENTS WITH PULMONARY PARACOCCIDIOIDOMYCOSIS.

Fornazin, M.C.; Mamoni, R.L.; Balthazar, A.; Quagliato Jr., R.; Blotta, M.H.S.L.

Faculdade de Ciências Médicas - UNICAMP, Campinas, SP, Brazil.

To investigate the local vs. systemic immune response we analyzed the phenotype of different cell population in peripheral blood and bronchoalveolar lavage (BAL) of patients with pulmonary paracoccidioidomycosis (PCM), with the focus on activation markers, adhesion molecules, and costimulatory molecules, as well as the cytokine production. The group consisted of 19 patients (16 male and 3 female) with age ranging from 34 to 65 years. Diagnosis was confirmed by demonstration of *P. brasiliensis* cells in the sputum, bronchoalveolar lavage fluid (BAL), or biopsy, in addition to serological tests. The lungs image study showed bilateral and diffuse infiltrates with nodules. Cytospin preparations from patients with PCM showed an increased number of lymphocytes and neutrophils in BAL, when compared with healthy subjects. The T lymphocyte population was constituted mainly by CD8+ cells, while in peripheral blood a predominance of CD4+ T cells was observed. The alveolar macrophages (AM) expression of class II, ICAM-1 and B7-2 molecules was significantly higher than in peripheral blood monocytes (PBM). The upregulated expression of these molecules in AM is indicative of a preserved and active macrophage function in patients with PCM. ICAM-1 (CD54) plays an important role in the extravasation of leukocytes and in other cellular functions such as cytoxicity, phagocytosis, chemotaxis and induction of lymphocyte proliferation. The activation molecule MHC-class II as well as B7-2 are essential for the macrophage-APC function. Cultured AM produced higher levels of IL-6, TNF-alpha, and MIP-1-alpha as compared with cultured PBM. No differences were detected in relation to IL-8, IL-12p40, IL-10 and TGF-beta ?production. BAL fluid from PCM patients also contained detectable levels of IL-6, TNF-alpha and MIP-1alpha. TNF-alpha has been shown to be a critical mediator of innate immunity against several respiratory pathogens. His activities include stimulation of neutrophils for enhanced protein release and respiratory burst, and enhancement of neutrophils phagocitic activity and killing. TNF-alpha is also required for macrophages accumulation and

differentiation into epithelioid cells and for the persistence of well-formed granulomas. A significant correlation was observed between the levels of MIP-1alpha produced by AM in culture (*ex vivo*) and the number of CD8+ T cells in BAL. MIP-1alpha (macrophage inflammatory protein) is a C-C chemokine induced during inflammation, by alveolar macrophages, gamma-delta T cells, NK cells and lung epithelium. MIP-1alpha was shown to promote chemotaxis of Th1 but not Th2 cells *in vitro*. Our finding of a positive correlation between MIP-1alpha production and the number of CD8+ T cells supports the notion that MIP-1alpha is important in attracting and stimulating CD8+Tcells. A protective role for CD8+ T cells was suggested in experimental PCM since its depletion induces a more severe and/or disseminated disease in both, resistant and susceptible mice. Cytotoxic CD8+ T cells represent a major defense against pathogens by the production of IFN-gamma and cytolytic activity and may be involved in the clearance of *P. brasiliensis* cells. In experimental cryptococcosis it was demonstrated that CD8+ T cells are required for maximal recruitment of CD4+ T cells into the lungs and IFN-gamma production, which play a role in macrophage activation and development of a protective Th1 type CD4+ T cell. In conclusion these findings indicate that the local inflammatory reaction in the lungs of patients with pulmonary paracoccidioidomycosis is mediated by the inflammatory cytokines TNF-alpha IL-6 and by the chemokine MIP-1alpha which may play an important role mediating the recruitment of lymphocytes (CD8+ T cells) and macrophages to the site of infection.