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PARACOCCIDIOIDOMYCOSIS: CHALLENGES IN THE DEVELOPMENT OF A VACCINE AGAINST AN ENDEMIC MYCOSIS IN THE AMERICAS

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SUMMARY

Paracoccidioidomycosis (PCM), caused by *Paracoccidioides* spp, is an important endemic mycosis in Latin America. There are two recognized *Paracoccidioides* species, *P. brasiliensis* and *P. lutzii*, based on phylogenetic differences; however, the pathogenesis and disease manifestations of both are indistinguishable at present. Approximately 1,853 (~51,2%) of 3,583 confirmed deaths in Brazil due to systemic mycoses from 1996-2006 were caused by PCM. Antifungal treatment is required for patients with PCM. The initial treatment lasts from two to six months and sulfa derivatives, amphotericin B, azoles and terbinafine are used in clinical practice; however, despite prolonged therapy, relapses are still a problem. An effective Th1-biased cellular immune response is essential to control the disease, which can be induced by exogenous antigens or modulated by prophylactic or therapeutic vaccines. Stimulation of B cells or passive transference of monoclonal antibodies are also important means that may be used to improve the efficacy of paracoccidioidomycosis treatment in the future. This review critically details major challenges facing the development of a vaccine to combat PCM.

KEYWORDS: *Paracoccidioides brasiliensis; Paracoccidioides lutzii;* Peptide P10; Vaccine; Immunoprotection.

REVIEW

Paracoccidioidomycosis (PCM), is a systemic mycosis with clinical manifestations of a granulomatous disease, caused by thermally dimorphic *Paracoccidioides* spp. Adolpho Lutz first described this fungus in 1908, while examining oral lesions in two patients[16]. PCM is the most frequent systemic endemic mycosis in Latin America, with the highest incidence of diagnosis in Brazil, Argentina, Colombia, and Venezuela[23]. The main route of infection is the inhalation of fungal particles, which usually leads to an asymptomatic infection[4]. There are two main clinical forms of PCM, acute/subacute and chronic. The acute/subacute form is characterized by a rapid disease course (weeks to months), impaired cellular immunity, an absence of delayed-type hypersensitivity reactions and a high mortality rate. The chronic form affects mainly 30-50 years old males with disease manifestations that are predominant pulmonary and/or mucocutaneous[13]. In terms of mortality, based on data obtained from the Brazilian Ministry of Health´s Mortality Information, approximately 1,853 (~51,2%) of 3,583 confirmed deaths in Brazil due to systemic mycoses from 1996-2006 were caused by PCM[22].

Expanding on the description of *P. brasiliensis* by Adolpho Lutz, modern molecular studies analyzing the genetic variability of strains revealed the existence of three distinct phylogenetic groups, namely: S1 - paraphyletic group with isolates from Argentina, Brazil, Peru and Venezuela; PS2 - monophyletic group with isolates from Brazil and Venezuela; and PS3 - monophyletic group with Colombian isolates only[30]. Subsequently, a complementary comparative genomic study identified an isolate that was separated from the other groups distributed on the *Paracoccidioides* cladogram[8]. This analysis led to a re-classification of this isolate as a new species within the genus, named *Paracoccidioides lutzii*[29], which is now known to be endemic in the North and Central-West regions of Brazil (States of Rondonia, Mato Grosso and Goias) and geographically partially overlaps with group S1. Despite the genetic differences, the pathogenesis and disease manifestations of *P. brasiliensis* and *P. lutzii* are indistinguishable at present. One important difference, is that *P. lutzii* does not properly express a key glycoprotein, gp43[30], which is a target of vaccine development detailed below.

Antifungal chemotherapy is required for clinical PCM, although there is no certainty of total elimination of the fungus at the end of treatment. Initial treatment lasts from two to six months based on the extent of disease and clinical response to therapy, and typically includes the use of sulfa derivatives (sulfadiazine, sulfadoxine, sulfamethoxypyridazine, cotrimazine and trimethoprim-sulfamethoxazole) although amphotericin B, azoles (ketoconazole, itraconazole, fluconazole, voriconazole and posaconazole) or terbinafine may also be used. After the initial intensive therapy, extended periods of treatment are often necessary, up to two or more years, with a significant frequency of relapsing disease[3,26].

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Protection against PCM has been attributed to the induction of cellular immune responses whereas high levels of specific antibodies have been associated with the symptomatic form of the disease. A major line of investigation has focused on purified antigens in the attempt to develop a peptide vaccine. The glycoprotein gp43 is the main antigen target of *P. brasiliensis* and a 15-mer internal peptide (QTLIAIHTLAIRYAN), known as P10, contains the major $CD4^+$ specific T cell epitope and elicits an IFN-γ-dependent Th1 immune response. Immunization with P10 of intratracheally infected BALB/c mice, in the presence of complete Freund adjuvant (CFA) reduces the fungal burden in the lungs, liver and spleen[28,32].

The protection by P10 administered in CFA[18] observed in a prophylactic protocol was also obtained therapeutically in *P. brasiliensis*-infected immunocompetent mice. Similar results were observed in partially immunosuppressed mice[21], in which case fibrosis was also precluded.

Complete Freund adjuvant causes a variety of side effects such as localized injection-site granulomas, hepatic and renal granuloma formation and necrotizing dermatitis. Hence, the use of this adjuvant has been banned in humans as well as in non-experimental veterinary administration[27]. With this in mind, our group tested the therapeutic effects of P10 combined with different adjuvants (aluminum hydroxide, CFA, flagellin and the cationic lipid dioctadecyl-dimethylammonium bromide (DODAB)) in BALB/c mice previously infected with *P. brasiliensis*[19]. Seventy days after infection, mice treated with DODAB and P10, and with less intensity, mice treated with P10 and flagellin, showed the most prominent effects. Concomitantly, secretion of IFN-γ and TNF-α, in contrast to IL-4 and IL-10, was enhanced in the lungs of mice immunized with P10 in combination with the tested adjuvants, with the best results observed in mice treated with P10 and DODAB[19].

The incorportion of P10 in Poly (lactic acid-glycolic acid) nanoparticles (PLGA) at 1 μg/50μL with TMP/SMZ was also tested. It reduced the amount of peptide necessary to decrease the fungal load in the infected animals and avoid disease relapse when compared with P10 emulsified in Freund's adjuvant (20 μg/50μL)[2].

The use of pcDNA3 expression vector encoding P10 was a gene therapy approach tested in intratracheally infected mice. The plasmid vaccine induced a significant reduction of fungal burden in the lung. Co-vaccination with a plasmid encoding mouse IL-12 proved to be even more effective in the elimination of the fungus with virtual sterilization in a long term (five months) infection and treatment assay[25]. The immunization with plasmid encoding P10 was also able to induce memory cells[6].

Dendritic cells (DCs) pulsed with P10 protected mice infected with *P. brasiliensis*. The adoptive transference of pulsed dendritic cells in mice previously infected with the fungus reduced the fungal burden in their lungs[17]. FERREIRA *et al*., (2011)[12] used DCs transfected with plasmid (pMAC/PS-scFv) encoding a single chain variable fragment (scFv) of an anti-Id antibody that was capable of mimicking gp43. Mice immunized subcutaneously with pMAC/PS-scFv decreased the lung infection.

Other peptides from 43 kDa glycoprotein showed different properties such as P4 and P23 that inhibit macrophage functions and the inflammatory reaction, thus facilitating infection[14,15]. P4 and P23 were evaluated in different models *in vivo*, and suggested that this anti-inflammatory effect depended on the kinetics of innate immunity modulators such as TNF-α, IL6, IL10 and TLR2. IL10 seems to be produced earlier than TNF-α and IL6, in presence of the peptides. The anti-inflammatory effects of P4 and P23 depended on the amount and frequency of treatment[15].

Besides the use of gp43 and P10 as vaccines, other antigens have been investigated as additional vaccine options against *P. brasiliensis*. cDNA encoding the 27 kDa protein present at the surface and cytosol of *P. brasiliensis* was subcloned into a pET-DEST 42 plasmid and expressed in *Escherichia coli* (rPb27). BALB/c mice were infected with virulent *P. brasiliensis* and after being immunized subcutaneously with purified rPb27 in the presence of *Corynebacterium parvum* and aluminum hydroxide, some mice were also treated with fluconazol. After 40 days of treatment, the combined administration of plasmid and chemotherapeutics controlled PCM in the lung, liver and spleen[10,11]. A therapeutic study was conducted to evaluate fibrosis development in animals immunized with rPb27 and infected. After 30 and 90 days post-infection reduced levels of collagen and receptor CCR7 were observed with high levels of active caspase 3, IFN-γ, TGF-β and IL-10 on the early phase of infection. In the control groups that developed high levels of pulmonary fibrosis, the molecule could be promising as a prophylactic and therapeutic treatment against PCM[20]. The use of rPb40 together with rPb27, combined with conventional treatment, exhibited additive protective effect[10].

Recombinant paracoccin (the sequence matched a hypothetical protein encoded by PADG-3347 of *P. brasiliensis* 18, with a polypeptide sequence similar to endochitinase) expressed in *E. coli* cells showed protective effect in infected mice reducing the fungal burden[1]. Otherwise, radioattenuated yeast cells of *P. brasiliensis* reduced the fungal burden in infected mice[9]. DNAhsp65 (Heat shock protein from *Mycobacterium leprae*) administered by intramuscular immunization in BALB/c mice promoted an increase in Th-1 cytokines and reduced the fungal burden[24].

The role of antibody-mediated immunity in PCM has been less certain as in early studies it was believed that antibodies were useful only for serological diagnosis. More recently, monoclonal antibodies (mAbs) have been shown to significantly modify PCM. The first demonstration of this utilized mAbs against gp70 of *P. brasiliensis*[7]. In fact, both protective and non-protective mAb have been identified as exemplified by a panel of mAbs recognizing gp43, of which mAb 3E was the most efficient in reducing the fungal burden *in vivo* and promoting fungal phagocytosis *in vitro*[5]. MAb 3E recognized the epitope sequence NHVRIPIGYWAV. Significantly, combining P10 preimmunization with the administered of mAb 3E 24 h before intratracheal challenge with virulent *P. brasiliensis* yeasts resulted in additive protection using a short-term protocol in comparison with a non-protective mAb[32].

The mechanisms for protection against *P. lutzii* are not well elucidated. We recently demonstrated that mAbs generated against the heat shock protein 60 (Hsp60) from *Histoplasma capsulatum* interacted with *P. lutzii* yeast cells and enhanced phagocytosis by macrophages cells[31]. The passive transfer of Hsp60-binding mAbs 7B6 and 4E12 significantly reduced the lung fungal burden in BALB/c mice intratracheally infected with *P. lutzii.*

Although we currently have several therapeutic options for the treatment of PCM, these regimens require protracted administration of antifungal drugs, increasing both the costs of therapy and the incidence of toxicities. Moreover, there are frequent relapses despite more than one or two years to antifungal treatment. In light of this, efforts are underway to harness the host immune system to generate protective responses that can improve the efficacy and reduce the duration of treatment. The use of peptides, purified antigens, DNA therapy, peptide-pulsed DCs, radio attenuated yeast cells, monoclonal antibodies, etc. are being explored as potential components of a therapeutic vaccine. To date, these promising studies have provided results in experimental model or *in vitro* in patients' cells. We are now poised to transition the large amount of knowledge gained through these studies into clinical trials aimed at improving our ability to combat PCM.

RESUMO

Paracoccidioidomicose: desafios no desenvolvimento de uma vacina contra micose endêmica nas Américas

A paracoccidioidomicose (PCM), causada por *Paracoccidioides* spp, é importante micose endêmica na América Latina. Com base em diferenças filogenéticas, existem duas espécies reconhecidas de *Paracoccidioides, P. brasiliensis* e *P. lutzii*, no entanto, a patogênese e as manifestações clínicas de ambas são indistinguíveis atualmente. Aproximadamente 1853 (~51,2%) de 3583 mortes confirmadas, atribuídas a micoses sistêmicas de 1996-2006, no Brasil foram causadas por PCM. Tratamento antifúngico é necessário para pacientes com PCM. O tratamento inicial dura de dois a seis meses e derivados de sulfa, anfotericina B, azóis e terbinafina são utilizados na prática clínica; no entanto, apesar da terapêutica prolongada, as recaídas ainda são um problema. Uma resposta imune celular eficaz, tendendo a Th1, é essencial para controlar a doença que pode ser induzida por antígenos exógenos, ou moduladas por vacinas profiláticas ou terapêuticas. A estimulação de células B ou a transferência passiva de anticorpos monoclonais também são meios importantes que podem ser utilizados para melhorar a eficácia do tratamento da paracoccidioidomicose no futuro. Esta revisão detalha criticamente os principais desafios que o desenvolvimento de uma vacina para combater a PCM enfrenta.

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