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HSP65 DNA as therapeutic strategy to treat experimental paracoccidioidomycosis

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ABSTRACT

The conventional treatment for paracoccidioidomycosis, the most prevalent mycosis in Latin America, involves long periods of therapy resulting in sequels and high frequency of relapses. The search for new alternatives of treatment is necessary. Previously, we have demonstrated that the *hsp*65 gene from *Mycobacterium leprae* shows prophylactic effects against murine paracoccidioidomycosis. Here, we tested the DNAhsp65 immunotherapy in BALB/c mice infected with *Paracoccidioides brasiliensis*, the agent of paracoccidioidomycosis. We observed an increase of Th1 cytokines accompanied by a reduction in fungal burden and pulmonary injury. These results provide new prospects for immunotherapy of paracoccidioidomycosis and other mycoses.

1. Introduction

Paracoccidioidomycosis (PCM) is a health problem in Latin America where an estimated 10 million individuals may be infected by its etiological agent, the dimorphic human pathogenic fungus *Paracoccidioides brasiliensis* [1]. PCM is the most prevalent systemic endemic mycosis in South America notably in Brazil, Colombia, Venezuela, and Argentina [2]. The fatal acute PCM affects the reticule endothelial system, whereas the chronic PCM affects mainly the lung, which shows a granulomatous inflammation with an inefficient cellular immune response [1,3].

The conventional therapy for PCM is based on sulphanamides, amphotericin B and azole derivatives [4]. Long-term therapy is usually required to warrant a good clinical response and avoid relapses. However, the treatment has its limitations – for example, high toxicity, low efficiency and drug resistance, mainly because of the growing number of immunocompromised patients. Thus, despite the existing antifungal drugs for treating patients with PCM, there is a demand for new therapeutical interventions that could help the clinicians to approach severely ill patients with an impaired host cell response, who usually have a poor and/or late response to conventional antifungal therapy.

Because of the escalating number of immunocompromised patients, such as those infected with HIV or undergoing anticancer therapy, the cases of resistance are increasing, which calls for even higher doses of the antifungal agents in use today. Higher dosages of some antifungal agents may cause serious toxic side effects, such as nephrotoxicity [5]. For that reason, alternative therapies are being studied using candidate antigen molecules and its mechanisms of protection against various fungi, including *P. brasiliensis* [6]. Among these molecules, heat-shock proteins (HSPs) represent attractive candidates due to their association with both innate and adaptive immunity [7]. HSP molecules have been applied into DNA- or protein (peptide)-based vaccines as antigens, chaperones or adjuvants [8]. Numerous members of this family of proteins have been tested for prophylaxis or immunotherapy against a great variety of illnesses, for instance, tumors, autoimmune diseases, and several types of infections, including mycosis [9–11].

In both human and experimental fungal infection, cell-mediated immunity is critical for host defense [12]. The successful resolution of infection with *P. brasiliensis* is dependent on the activation of cellular immunity. The immune response towards a preferential Th1 activation, with IFN-γ production and efficient macrophage activation is able to contain fungal dissemination and disease progression [13].

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In animal models, DNA vaccines have been successfully used against various pathogens, such as protozoa (*Trypanosoma cruzi* [14], *Leishmania major* [15] and *Giardia lamblia* [16], viruses (hepatitis B) [17], mycobacteria (*Mycobacterium tuberculosis*) [18]. Current studies indicate that the HSPs induce preferentially a cellular immune response [19] and HSP vaccines can be used for cancer immunotherapy [20] as well as for the treatment of tuberculosis [21].

Several studies have demonstrated a potential use of HSP65 from *Mycobacterium leprae* as an immunomodulator. A DNA vaccine encoding for the protein HSP65 (DNAhsp65) was able to confer protection to mice and guinea pigs against *M. tuberculosis* challenge [22]. Other experiments showed even better results when DNAhsp65 and drug treatments were combined [21]. In both cases, therapeutic effects appeared to be associated with the priming of a strong Th1 immune response and down-regulation of Th2 cytokines. More importantly, this response not only reduced bacilli loads in the lungs but also conserved the histological structure of the lung parenchyma [22]. Our previous results have shown that a DNAhsp65 vaccine protected mice against a virulent strain of *P. brasiliensis*, the etiological agent of the PCM [6].

In the present study, we used the DNAhsp65 plasmid to treat mice infected with the virulent *P. brasiliensis*. The immunomodulation and the ability to elicit specific cellular and humoral immunity by DNAhsp65 as well as its use as immunotherapeutic were investigated.

2. Methods

2.1. DNAhsp65 plasmid construction and purification

The plasmid DNAhsp65 was derived from the vector pVAX1 vector (Invitrogen, Carlsbad, CA, USA), which had previously been digested with BamHI and NotI (Invitrogen), and a fragment of the *M. leprae hsp*65 gene was inserted. The empty pVAX vector was used as a control. Plasmid DNA was obtained from transformed DH5α *E. coli* cultured in LB liquid medium (Gibco BRL, Gaithersburg, MD, USA) containing kanamycin (50 μg/mL). The plasmids were purified using the Endofree Plasmid Mega kit (Qiagen, Valencia, CA, USA). DNA concentration was estimated spectrophotometrically at 260 and 280 nm using the Gene Quant II apparatus (Pharmacia Biotech, Buckinghamshire, UK). Endotoxin levels were determined using a QCL-1000 Limulus amoebocyte lysate kit (Cambrex Company, Walkersville, MD, USA) and were less than 0.1 endotoxin units (EU)/μg, as recommended by European and US Pharmacopoeias. The purity of DNA preparations was verified by electrophoresis (1% agarose).

2.2. Mice

Male BALB/c mice (6–8-week old) were obtained from University of São Paulo (Ribeirão Preto Campus, SP, Brazil) and maintained under standard laboratory conditions. All experiments involving animals were approved by Bioethical Committee of University of Brasília (UnB, Brasília, DF, Brazil) and conducted in accordance with their guidelines.

2.3. P. brasiliensis strain and infection

The yeast form of the virulent *P. brasiliensis* strain 18 [23] was used for the infection assays. The fungus was cultured in liquid YPD medium (w/v: 2% peptone, 1% yeast extract, 2% glucose) at 36 °C in a rotary shaker (220 rpm) for 5 days. After this period, a suspension of *P. brasiliensis* cells was prepared at a concentration of 10^7 viable cells/mL. Viability was determined through the Janus Green B vital dye method [24] (Merck, Darmstadt, Germany). It was found to be higher than 80%.

To infect the mice intravenously, 100 μL of this suspension containing 10^6 cells was inoculated per animal. The chosen route of infection was intravenous because this is the established model of stimulating systemic and chronic diseases and that is the situation we intended to analyze with respect to the effectiveness of the DNAhsp65 plasmid therapy.

2.4. Treatment

After 30 days of infection, the treatment was initiated by intramuscular route. Mice were divided into four groups (10 animals per group): group I – mice non-infected and non-treated (non-infected group); group II – mice infected and treated with saline 0.9% (infected group); group III – mice infected and treated with 4 doses of 100 μg of empty vector pVAX1 at 2-week intervals (pVAX1 group); group IV – mice infected and treated with 4 doses of pVAX1-*hsp*65 at 2-week intervals (DNAhsp65 group). The animals were euthanized by cervical dislocation 15 days after the last treatment dose. Lung, spleen, liver and serum samples were collected for sequential analysis.

2.5. Histopathology and determination of P. brasiliensis CFU in lungs

To evaluate lesion progress, lung tissue fragments were fixed in 10% formalin for 6 h, dehydrated in alcohol, and embedded in paraffin. Serial 5-μm sections were stained either with hematoxylin–eosin (HE) to visualize the fungus and granulomatous appearances or with Masson's trichrome to detect collagen fibers.

Lung fungal burdens were measured by quantitative counts of colony-forming units (CFU) of *P. brasiliensis*. Lung, spleen and liver fragments were homogenized in 1.0 mL of PBS (pH 7.2). Aliquots of 100 μL of these homogenates were plated onto brain heart infusion agar (BHI agar), supplemented with 4% horse serum, 5% *P. brasiliensis* 192 (Pb192) culture filtrate [25] and gentamycin 40 mg/L (Gentamycin Sulfate, Schering-Plough, Rio de Janeiro, Brazil). The Pb192 culture filtrate was prepared as previously described [25]. The plates were incubated at 36 °C, and the number of colonies was counted after incubation for 21 days. Results were expressed as number of CFU ± standard error of the mean (S.E.M.) per gram of lung tissue.

2.6. Cell proliferation assay and measurement of cytokines concentration

T-cell proliferative responses to concanavalin-A (ConA) were studied by [^{3}H]-thymidine incorporation as previously described [26]. Spleen cells were disrupted in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 5% non-essential amino acids (Sigma Chemical Co., St. Louis, MO), 2 M streptomycin (100 μg/mL), and 5% fetal bovine serum (FBS). Cells were washed twice in serum-free RPMI, counted, added to 96-well plates at a cell density of 3×10^5 cells/well and subsequently stimulated with ConA (4 μg/mL, Sigma Chemical Co., St. Louis, MO). The experiments were performed in triplicate at a final volume of 200 μL/well. After 48 h of incubation at 37 °C under 5% CO_2, cultures were pulsed for 12 h with 1 μCie/well of with H^3-labeled thymidine (Amersham, Arlington Heights, IL) and then harvested. The incorporation of H^3-thymidine was measured using a Liquid Scintillation Counter (Beckman Instruments); the data were expressed as means ± S.E.M. of counts per minute of H^3-thymidine incorporation.

Supernatants of spleen cells cultured from experimental groups were used for detection of cytokines production. The cytokines