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A cell wall protein-based vaccine candidate induce protective immune response against *Sporothrix schenckii* infection

Deivys Leandro Portuondo[a], Alexander Batista-Duharte[b], Lucas Souza Ferreira[a], Damiana Téllez Martínez[a], Marisa Campos Polesi[a], Roberta Aparecida Duarte[a], Ana Carolina Alves de Paula e Silva[a], Caroline Maria Marcos[a], Ana Marisa Fusco de Almeida[a], Iracilda Zeppone Carlos[a,*]

[a] *Department of Clinical Analysis, Araraquara's School of Pharmaceutical Sciences, Universidade Estadual Paulista-UNESP, Júlio Mesquita Filho, Rua Expedicionários do Brasil, 1621, Postal Code: 14801-902, Araraquara, SP, Brazil*

[b] *Immunotoxicology Laboratory, Toxicology and Biomedicine Center (TOXIMED), Medical Science University, Autopista Nacional Km. 1 1/2CP 90400, AP 4033 Santiago de Cuba, Cuba*

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ABSTRACT

Sporotrichosis is a subcutaneous mycosis caused by several closely related thermo-dimorphic fungi of the *Sporothrix schenckii* species complex, affecting humans and other mammals. In the last few years, new strategies have been proposed for controlling sporotrichosis owning to concerns about its growing incidence in humans, cats, and dogs in Brazil, as well as the toxicity and limited efficacy of conventional antifungal drugs. In this study, we assessed the immunogenicity and protective properties of two aluminum hydroxide (AH)-adsorbed *S. schenckii* cell wall protein (ssCWP)-based vaccine formulations in a mouse model of systemic *S. schenckii* infection. Fractioning by SDS-PAGE revealed nine protein bands, two of which were functionally characterized: a 44 kDa peptide hydrolase and a 47 kDa enolase, which was predicted to be an adhesin. Sera from immunized mice recognized the 47 kDa enolase and another unidentified 71 kDa protein, whereas serum from *S. schenckii*-infected mice recognized both these proteins plus another unidentified 9.4 kDa protein. Furthermore, opsonization with the anti-ssCWP sera led to markedly increased phagocytosis and was able to strongly inhibit the fungus' adhesion to fibroblasts. Immunization with the higher-dose AH-adjuvanted formulation led to increased *ex vivo* release of IL-12, IFN-γ, IL-4, and IL-17, whereas only IL-12 and IFN-γ were induced by the higher-dose non-adjuvanted formulation. Lastly, passive transference of the higher-dose AH-adjuvanted formulation's anti-ssCWP serum was able to afford *in vivo* protection in a subsequent challenge with *S. schenckii*, becoming a viable vaccine candidate for further testing.

1. Introduction

Diseases caused by opportunistic fungi are an increasing health problem, especially for immunocompromised individuals. Sporotrichosis, also known as "rose gardener's disease", is a subcutaneous mycosis caused by several closely related thermo-dimorphic fungi of the *Sporothrix schenckii* species complex, including *Sporothrix brasiliensis*, *Sporothrix globosa, Sporothrix mexicana, Sporothrix luriei* and *S. schenckii sensu stricto* (Oliveira et al., 2014). The disease is commonly found in tropical and subtropical regions, but isolated cases and outbreaks have been reported worldwide (Rodrigues et al., 2013). *S. schenckii* is a ubiquitous envi-

Abbreviations: ssCWP, *S. schenckii* cell wall protein; AH, aluminum hydroxide; gp70, glycoprotein of 70 kD; PMSF, phenylmethyl sulfonyl fluoride; EDTA, Ethylenediamine tetraacetic acid; BCA, bicinchoninic acid; DTT, dithiothreitol; FA, formic acid; ACN, acetonitrile; FBS, fetal bovine serum; NIS, serum from non-immunized mice; IS, serum from *S. schenckii*-infected mice; MFI, median fluorescence intensity; ADCC, antibody-dependent cellular cytotoxicity.

* Corresponding author: Department of Biosciences and Biotechnology Applied to Pharmacy, Araraquara's School of Pharmaceutical Sciences, Universidade Estadual Paulista–UNESP,Júlio Mesquita Filho, Rua Expedicionários do Brasil, 1621, Postal Code: 14801–902, Araraquara, SP, Brazil. Fax: +55 16 3332 0880.

E-mail addresses: deivysleandro@gmail.com (D.L. Portuondo), batistaduhartea@gmail.com (A. Batista-Duharte), gigabreath@hotmail.com (L.S. Ferreira), damianatellezm@gmail.com (D.T. Martínez), marisapolesi@yahoo.com.br (M.C. Polesi), robduarte@hotmail.com (R.A. Duarte), ana_alpasi@hotmail.com (A.C.A. de Paula e Silva), marcos_caroline@yahoo.com.br (C.M. Marcos), almeidaf@fcfar.unesp.br (A.M.F.d. Almeida), deivysleandro@gmail.com (I.Z. Carlos).

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ronmental saprophyte that can be isolated from soil and plant debris, normally causing infection by its traumatic inoculation from contaminated material (Barros et al., 2011). In Brazil, sporotrichosis has become an important zoonosis, with cats being the main source of infection and transmitting agent to humans and other animals (Rodrigues et al., 2013). Cat-transmitted sporotrichosis represents 91% of all cases of human sporotrichosis in Brazil (Freitas et al., 2010), which in great part due to the presence of a large number of viable yeasts on the surface of feline ulcerated lesions or by inoculation of the fungus following cat scratches (Cruz, 2013). Sporotrichosis' most frequent clinical manifestations are the localized or regional lymphocutaneous forms, while the disseminated form has been mainly reported among immunocompromised individuals (Barros et al., 2011).

The usual sporotrichosis treatment requires long periods of antifungal drug administration accompanied by frequent relapses in immunocompromised patients (Kauffman et al., 2007). This therapy is often associated with sometimes severe adverse effects and frequent fungal resistance (Rodrigues et al., 2014). Therefore, vaccination has been proposed as a viable alternative for both therapeutic and prophylactic purposes (Almeida, 2012; Lacerda et al., 2011). For decades, a variety of cell wall proteins (CWPs) from many different pathogenic fungi have been evaluated in mouse models of vaccination for assessment of their immunogenicity, safety and protection-affording potential (Edwards, 2012). Several authors have reported the development of a specific immune response and increased resistance to subsequent infection following either a previous infection or active immunization with *S. schenckii* CWPs (ssCWPs) or whole cells (Charoenvit and Taylor, 1979; Tachibana et al., 1999).

Studies performed in our lab have shown that immunization with dendritic cells stimulated with *S. schenckii* yeasts or their exoantigen was able to induce a Th1 and Th17 mixed response *in vitro*(Verdan et al., 2012), the latter of which has been since associated with control of the *S. schenckii* infection *in vivo* (Ferreira et al., 2015). Also, Nascimento and Almeida (2005) reported induction of a specific humoral response against the 70 kDa glycoprotein (gp70), a key immunodominant antigen of the *S. schenckii* cell wall, in a mouse model of infection. This antigen's relevance was confirmed by studies in which transference of anti-gp70 mAbs was able to convey protection against highly virulent *S. schenckii* and *S. brasiliensis* strains, thus providing definitive evidence for the role of antibodies in the protective immunity against *S. schenckii* (de Almeida et al., 2015). Although very few clinical trials have been performed in humans, a growing number of antifungal vaccine candidates are being evaluated in preclinical studies, as part of the renewed interest in the potential use of vaccines, replacing or associated with chemotherapy, to reduce antifungal drugs use and consequently limit drug resistance and toxicity (Portuondo et al., 2015).

In this study, we assessed the immunogenicity and protective properties of two AH-adsorbed ssCWP-based vaccine formulations in a mouse model of systemic *S. schenckii* infection. Our results showed the vaccine formulations to be immunogenic and able to promote protection-affording antibody production upon immunization.

2. Materials and methods

2.1. Animals

Male 5–7 week-old BALB/c mice were purchased from "Centro Multidisciplinar para Investigação Biológica na Área da Ciência de Animais de Laboratório" (CEMIB), UNICAMP University (Brazil) and maintained under standard laboratory care as previously described (Goncalves et al., 2015). This work was approved by the Institutional Ethics Committee for Animal Use in Research (Protocol CEUA/FCF/CAR no. 30/2012) and was in accordance with the National Institutes of Health Animal Care guidelines.

2.2. Microorganism and culture conditions

S. schenckii ATCC 16345 was kindly provided by the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. The isolate was maintained by regular passage in mice and grown on Mycosel™ (BD Biosciences) agar tubes at 25 °C. Mycelial-to-yeast phase conversion was accomplished as previously described (Ferreira et al., 2015).

2.3. Extraction of the ssCWPs

Extraction of the ssCWPs was performed as previously described (Castro et al., 2013), with minor modifications. Briefly, yeast cells collected from logarithmically growing cultures were incubated with the dithiothreitol (DTT)-based protein extraction buffer (2 mM DTT, 1 mM phenylmethyl sulfonyl fluoride, and 5 mM EDTA in This/HCl buffer) for 2 h at 4 °C under mild agitation. The ssCWP-containing supernatant was collected, dialyzed against distilled water, filtered through a 0.22 μm nitrocellulose membrane (Millipore) and then concentrated using the Amicon Ultra 15 MWCO concentrator (Millipore). The proteins were then precipitated by overnight incubation with 10% (w/v) trichloroacetic acid in acetone at 4 °C and the resulting pellets were washed in ice-cold acetone, dried in a SpeedVac® and reconstituted in phosphate buffered saline, pH 7.2–7.4 (hereafter referred to as PBS only). Protein concentration was measured by the BCA assay (Pierce).

2.4. Yeast viability assay

The effect of DTT protein extraction on *S. schenckii*'s viability was assessed using the LIVE/DEAD® yeast viability kit (Molecular Probes) in accordance with the manufacturer's instructions. Live DTT-untreated or heat-killed *S. schenckii* yeasts were used as the negative or positive controls, respectively. Samples were visualized on the BH50 fluorescence microscope (Olympus) using the fluorescein and DAPI filters for the FUN1 and Calcofluor White M2R stains, respectively.

2.5. One-dimensional sodium dodecyl sulfate-polycrylamide gel electrophoresis (SDS-PAGE) and MALDI-TOF mass spectrometry analysis

Samples containing 50 μg of the ssCWPs were separated by SDS-PAGE (10%) as described by Laemmli (1970). Protein spots excised from silver-stained gels were reduced, alkylated, and subjected to tryptic digestion with trypsin (Promega). Next, tryptic peptides were extracted and submitted to mass spectrometry (MS) analysis; results were collected with the AB Sciex Maldi TOF/TOF series explorer version 4.1.0 and data was analyzed in the Protein Pilot Software using the MASCOT search engine. The obtained sequences were compared to those in the SwissProt databanks (http://expasy.org/sprot). Additionally, prediction of adhesin-like proteins was performed using the FungalRV database.

2.6. Adsorption studies

Aluminum hydroxide (AH) gel (Invivogen) containing 10 mg/mL of Al^{3+} was used in the present work. The equilibrium time for adsorption of the ssCWPs on AH was determined by mixing 1 mg of the ssCWPs with an amount of AH corresponding to 1 mg of AL^{3+} to a final volume of 1 mL in PBS. The suspensions were kept under agitation on a rotary spinner at room temperature (RT) and pH 7.4 for 10–100 min. The amount of adsorbed protein