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Comparative efficacy and toxicity of two vaccine candidates against *Sporothrix schenckii* using either Montanide™ Pet Gel A or aluminum hydroxide adjuvants in mice

Deivys Leandro Portuondo [a], Alexander Batista-Duharte [a], Lucas Souza Ferreira [a], Cleverton Roberto de Andrade [b], Camila Quinello [a], Damiana Téllez-Martínez [a], Maria Luiza de Aguiar Loesch [a], Iracilda Zeppone Carlos [a,*]

[a] São Paulo State University (UNESP), School of Pharmaceutical Sciences, Department of Clinical Analysis, Araraquara, SP, Brazil
[b] São Paulo State University (UNESP), School of Dentistry, Department of Physiology & Pathology, Araraquara, SP, Brazil

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ABSTRACT

Sporotrichosis is an important zoonosis in Brazil and the most frequent subcutaneous mycosis in Latin America, caused by different *Sporothrix* species. Currently, there is no effective vaccine available to prevent this disease. In this study, the efficacy and toxicity of the adjuvant Montanide™ Pet Gel A (PGA) formulated with *S. schenckii* cell wall proteins (ssCWP) was evaluated and compared with that of aluminum hydroxide (AH). Balb/c mice received two subcutaneous doses (1st and 14th days) of either the unadjuvanted or adjuvanted vaccine candidates. On the 21st day, anti-ssCWP antibody levels (ELISA), the phagocytic index, as well as the *ex vivo* release of IFN-γ, IL-4, and IL-17 by splenocytes and IL-12 by peritoneal macrophages were assessed. Cytotoxicity of the vaccine formulations was evaluated *in vitro* and by histopathological analysis of the inoculation site. Both adjuvanted vaccine formulations increased anti-ssCWP IgG, IgG1, IgG2a, and IgG3 levels, although IgG2a levels were higher in response to PGA +CWP100, probably contributing to the increase in *S. schenckii* yeast phagocytosis by macrophages in the opsonophagocytosis assay when using serum from PGA+CWP100-immunized mice. Immunization with AH+CWP100 led to a mixed Th1/Th2/Th17 *ex vivo* cytokine release profile, while PGA+CWP100 stimulated a preferential Th1/Th2 profile. Moreover, PGA+CWP100 was less cytotoxic *in vitro*, caused less local toxicity and led to a similar reduction in fungal load in the liver and spleen of *S. schenckii*- or *S. brasiliensis*-challenged mice as compared with AH+CWP100. These results suggest that PGA may be an effective and safe adjuvant for a future sporotrichosis vaccine.

1. Introduction

Sporotrichosis is an emergent subcutaneous mycosis in tropical and subtropical regions, with isolated cases and outbreaks reported worldwide [1,2]. The disease is caused by different *Sporothrix* species, which are ubiquitous environmental saprophytes that can be isolated from soil and plant debris. In the environment, they can increase their virulence and cause infections to humans and other animals upon traumatic lesions with contaminated materials [3]. Over the last years, the zoonotic transmission of sporotrichosis through the bite or scratch of sick cats and other animals became an important cause of concern, mainly in Brazil [4,5].

Several pathogenic *Sporothrix* species have been described, including *S. brasiliensis*, *S. globosa, S. mexicana, S. lurie*, and *S. schenckii sensu stricto* [6]. In Brazil, the most frequently involved species in the cat-human zoonotic transmission is *S. brasiliensis* [5]. Sporotrichosis can assume many clinical forms, including fixed cutaneous or lymphocutaneous and disseminated forms, the latter

Abbreviations: ssCWP, *S. schenckii* cell wall protein; AH, aluminum hydroxide; PGA, Montanide™ Pet GeL A; gp70, glycoprotein of 70 kD; NIS, serum from non-immunized mice.

* Corresponding author at: São Paulo State University – UNESP, School of Pharmaceutical Sciences, Department of Clinical Analysis, Rodovia Araraquara-Jaú – km 1, 14800-903 Araraquara, SP, Brazil.

E-mail addresses: deivysleandro@gmail.com (D.L. Portuondo), batistaduhartea@gmail.com (A. Batista-Duharte), gigabreath@hotmail.com (L.S. Ferreira), clevertonroberto72@gmail.com (C.R. de Andrade), caquinello@hotmail.com (C. Quinello), damianatellezm@gmail.com (D. Téllez-Martínez), ma_luizaloesch@hotmail.com (M.L. de Aguiar Loesch), carlosiz@fcfar.unesp.br (I.Z. Carlos).

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of which has been mainly reported in immunocompromised individuals [6,7]. Given the renewed epidemiological importance of sporotrichosis and difficulties associated with the conventional antifungal drugs, different strategies are being investigated for prevention and treatment of this disease [8,9].

Several immune mechanisms have been shown to play a role in resistance against *S. schenckii* [9-17], impelling the use of immunomodulation tools for the management of sporotrichosis. Previously, we studied two aluminum hydroxide (AH)-adsorbed *S. schenckii* cell wall proteins (ssCWP)-based vaccine formulations and demonstrated induction of a strong specific immune response in vaccinated mice [18]. Furthermore, sera from those mice conferred protection against *S. schenckii* infection after passive transference in non-vaccinated and non-infected mice. In that study, a local inflammatory reaction at the inoculation site of immunized mice was observed.

Local reactions and tumors at the inoculation site have been associated with alum-adjuvanted vaccines in genetically predisposed cats, ferrets, and dogs [19–22]. Montanide™ Pet Gel A (PGA) is a ready-to-disperse polymeric adjuvant designed to improve the safety and efficacy of vaccines for companion animals, especially in cats [23].

Considering the high prevalence of animal-to-human transmission and the predominance of the highly virulent species *S. brasiliensis* as the etiological agent in cats [5,24], the objective of this work was to compare PGA and AH regarding their safety and effectiveness in inducing a protective immune response against this species when formulated with ssCWP. This study will help us choose a better adjuvant for a future anti-*Sporothrix* veterinary vaccine.

2. Materials and methods

2.1. Animals

Male 5–7 week-old specific-pathogen-free (SPF) Balb/c mice were purchased from the Multidisciplinary Center for Biological Research (CEMIB), University of Campinas, São Paulo, Brazil. Five mice per group were housed in microisolator cages and maintained under SPF conditions. This work was approved by the Institutional Ethics Committee for Animal Use in Research (Protocol CEUA/FCF/CAR 30/2012) and was in accordance with the National Institutes of Health Animal Care guidelines.

2.2. Microorganism and growth conditions

Sporothrix schenckii ATCC 16345 sensu stricto, isolated from a patient with diffuse lung infection (Baltimore, USA), and *S. brasiliensis* 250, isolated from a feline sporotrichosis case in Brazil, were kindly provided by the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. Mycelial-to-yeast conversion of both isolates was performed in 100 ml of brain–heart infusion broth (Difco) for 7 days at 37°C with continuous agitation at 150 rpm [15]. After that, an aliquot containing 2×10^7 or 2×10^5 yeasts from *S. schenckii* or *S. brasiliensis*, respectively, was transferred to a fresh medium and cultured for 5 more days at the same conditions.

2.3. Extraction of ssCWP

ssCWP extraction was performed as previously described [18].

2.4. Adjuvants and vaccine formulation

Aluminum hydroxide (AH) gel adjuvant was bought from Invivogen (EUA); Montanide™ Pet Gel A, an adjuvant composed of a highly stable dispersion of microspherical particles of sodium polyacrylate in water, was kindly provided by SEPPIC (France). The AH-ssCWPs formulation was prepared by mixing 0.1 mg of ssCWPs with an amount of AH equivalent to 0.1 mg of Al^{3+} (AH+CWP100) in a total volume of 100 μl and an adsorption time of 40 min [18]. The same antigen amount was formulated with 5% PGA (PGA+CWP100), according to the manufacturer's instructions. In mice, the 0.1 mg dose of Al^{3+} corresponds approximately to the maximum approved dose for human vaccines [25].

2.5. In vitro cytotoxicity

Mice were intraperitoneally (i.p) injected with 3 ml of a 3% sodium thioglycollate (Difco) solution 3 days before euthanasia. Peritoneal cells were harvested, plated in 96-well plates (5×10^5 cells/ml) in complete RPMI-1640 medium (cRPMI) and incubated overnight. Non-adherent cells were removed and macrophages were incubated with 100 μL of cRPMI containing either CWP100, AH+CWP100, PGA+CWP100, AH (100 μg of Al^{3+}), or 5% PGA at 37°C in a 5% CO_2 atmosphere. cRPMI or NaOH 0.1 N were used as negative or positive controls, respectively. After 20 h, cytotoxicity was determined using the MTT assay [26].

2.6. Immunization schedule

Balb/c mice (n = 5) received two subcutaneous (s.c) injections (0.1 ml) on the back of the neck on days 0 and 14 with CWP100, AH+CWP100, PGA+CWP100, or PBS alone as negative control. Serum obtained 1 week after the second immunization was heat-inactivated at 56°C for 30 min, aliquoted and stored at −20°C for further use.

2.7. Serum antibody titration and opsonophagocytosis assay

Both assays were conducted as described by Portuondo et al. [18]. Shortly, serum levels of anti-ssCWP IgG, IgG1, IgG2a and IgG3 were measured by ELISA. For the opsonophagocytosis assay, thioglycollate-elicited peritoneal macrophages were co-cultured in a 1:4 ratio with opsonized or non-opsonized *S. schenckii* yeasts in LabTek® slides (Nunc) for 2 h at 37°C. After that, the slides were stained with Giemsa and phagocytic activity was expressed using the phagocytic index (mean number of phagocytosed yeasts per macrophage).

2.8. Flow cytometry analysis

Sporothrix brasiliensis yeasts obtained as described on item 2.2 and then washed thrice with PBS at 4°C, were incubated at 37°C for 1 h with anti-ssCWP sera (1/20) from AH+CWP100- or PGA+CWP100-immunized or non-immunized mice. After that, the yeasts were washed with PBS and incubated with FITC-conjugated rabbit anti-mouse IgG (Sigma-Aldrich) (1/50) for 1 h at room temperature (RT). After washing, the binding of serum antibodies to the surface of the yeasts was determined using a flow cytometer (BD Accuri C6, BD Biosciences).

2.9. Cytokine induction assay

Thioglycollate-elicited peritoneal macrophages and total splenocytes were harvested from immunized mice and cultured in cRPMI for 24 h at 37°C and 5% CO_2 in the presence of ssCWPs. Final concentrations were 2.5×10^6 cells/ml and 40 μg of ssCWPs/ml in cRPMI; concanavalin A (0.25 μg/ml) or *Escherichia coli* O111B lipopolysaccharide (10 μg/ml) were used as positive controls for macrophages or splenocytes, respectively; cRPMI alone was used as negative control. The following supernatant-accumulated

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