Assessment of immunogenicity and protective efficacy of *Microsporum canis* secreted components coupled to monophosphoryl lipid-A adjuvant in a vaccine study using guinea pigs

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ABSTRACT

Microsporum canis is the most common dermatophyte in pets and is of zoonotic importance but currently there is no effective vaccine available to prevent dermatophytosis. The aim of this work was to assess the immunogenicity and protective efficacy of secreted components (SC) from *M. canis* adjuvanted with the monophosphoryl lipid-A (MPLA), in a vaccine study using the guinea pig as an experimental model. Animals were vaccinated with either the SC adjuvanted with the MPLA, the MPLA adjuvant alone or PBS three times at two-week intervals, until 42 days prior to *M. canis* infection. A blind evaluation of dermatophytosis symptoms development and fungal persistence in skin was monitored weekly. The antibody response towards the SC and the levels of Interferon (IFN)γ and Interleukin-4 expressed in peripheral blood mononuclear cells were assessed along or at the end of the study period respectively. The animals that received MPLA had a significantly lower clinical score than those inoculated with PBS. However, no significant difference was observed between the guinea pigs vaccinated with the SC adjuvanted with the MPLA and those having received MPLA alone. The results also showed that vaccination induced a strong antibody response towards the SC and an increase in IFNγ mRNA level. Our results show that the MPLA adjuvant used in this vaccine study can induce *per se* a partial protection against a *M. canis* infection. Although they induce a delayed-type hypersensitivity reaction in guinea pigs, the SC do not confer a protection under the present experimental conditions.

KEYWORDS

Dermatophytes, *Microsporum canis*, vaccination, monophosphoryl lipid-A adjuvant

INTRODUCTION

Microsporum canis is a filamentous fungus that causes superficial mycoses in pet animals (Weitzman and Summerbell, 1995; Mignon and Monod, 2011; Moriello and DeBoer, 2012). It is the main agent of dermatophytosis in cat, its natural host (Mignon and Losson, 1997), and is responsible for a frequent zoonosis (Seebacher et al., 2008). Successful treatment of *M. canis* dermatophytosis includes the use of systemic and topical antifungal agents for at least five weeks, confinement of the infected pet until cured and environmental decontamination. This makes the disease expensive and time consuming to treat and, because of the highly contagious nature of the disease a major problem in any animal husbandry situation (Moriello, 2004; Carlotti et al., 2010; Moriello and DeBoer, 2012). Immunoprophylaxis would present an important alternative to current control measures (Lund and Deboer, 2008). Several commercial and experimental vaccines against dermatophytosis have been developed and tested. In some cases there were encouraging results (Elad and Segal, 1994, 1995; Milan et al., 2004; Westhoff et al., 2010) while in other no protective immunity was found (DeBoer and Moriello, 1994, 1995; DeBoer et al., 2002). The exception is a vaccine for bovine dermatophytosis (Bovilis® Ringvac Intervet, the Netherlands). This vaccine, containing an attenuated strain LFT-130 *Trichophyton verrucosum*, has dramatically reduced the prevalence of dermatophyte infections in cattle and zoonotic infections in humans (Gudding and Naess, 1986). In cats, several attempts have been made to develop vaccines using characterized antigens from *M. canis*. The protective efficacy of a crude exo-antigen and two recombinant proteases, the subtilisin rSub3, a fungal endopeptidase involved in adherence of *M. canis* to human and animal epidermis (Baldo et al., 2010; Bagut et al., 2012), and the metalloprotease rMep3 have been tested in experimentally induced *M. canis* infections in guinea pig with inconclusive results (Descamps et al., 2003; Vermout et al., 2004).

The development of safe and effective vaccines requires the use of both appropriate antigens and adjuvants. In dermatophytoses, the Th1 cellular immune response, associated with delayed-type hypersensitivity (DTH), appears to be correlated with clinical recovery and protection against reinfection (Almeida, 2008; Mignon et al., 2008). Consequently, the use of adjuvants promoting the development of a Th1 immune response appears to be of major importance in the set-up of an effective vaccine against dermatophytosis. The monophosphoryl lipid-A (MPLA) adjuvant, a toll-like receptor (TLR)-4 agonist, is able to promote a Th1 response (Thompson et al., 2005) and could favour a protective immunity in dermatophytoses.

Recently, the secreted components (SC) from *M. canis* were shown to be potent activators of feline polymorphonuclear neutrophils by inducing the production of pro-inflammatory cytokines (Cambier et al., 2013). The aim of this study was therefore to assess the protective efficacy of the *M. canis* SC adjuvanted with the MPLA, using the guinea pig as an experimental infection model.

MATERIALS AND METHODS

Animals

Eighteen pathogen-free three-month-old female Hartley strain guinea pigs (Charles River Laboratories, Wilmington, MA, USA) were used for the vaccine study. Four additional animals were used for DTH test. The guinea pigs were housed in group cages, however vaccinated and non-vaccinated controls were strictly separated during the study. This study was approved by the local ethics committee of University of Liège, (ethics protocol no. 1053).

Production of *M. canis* arthroconidia and secreted components

Arthroconidia were produced from *M. canis* strain IHEM 21239 (Institute of Hygiene and Epidemiology-Mycology, Brussels, Belgium) using a previously described protocol (Tabart et al., 2007). The concentration of arthroconidia per ml was 1×10^6 as determined by serial dilutions on Sabouraud's (Sab; 2% glucose/1% peptone) agar medium plates.

The *M. canis* SC were obtained after growing arthroconidia in liquid Sab medium for 5 days at 27 °C (Cambier et al., 2013). Culture supernatant, containing SC, was separated from fungal elements by centrifugation, concentrated by ultrafiltration on an Amicon cell (Millipore, Billerica, MA, USA) using a filtration membrane with a size threshold of 10 kDa, dialyzed against 0.01 M PBS and stored at –20 °C until use. Protein concentrations were determined using the Bradford assay. The SC were subjected to 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using the method of Laemmli to determine the protein profile (Fig. 1). Liquid Sab medium (negative control) was also subjected to ultrafiltration and dialysis against 0.01 M PBS.

DTH test

Skin tests were performed in two guinea pigs 50 days after experimental infection and in two non-infected control animals. Ten µg of SC (100 µL) were injected intradermally at two sites on the flanks of animals. Both a negative (100 µL of liquid Sab medium) and a positive control consisting of 10 µg/100µL of a *M. canis* antigen known to induce DTH in immune guinea pigs (Mignon et al., 1999) were also performed. The skin thickness was measured before and 24 h after injection with a micrometre gauge (Kulche Coppieters, Brussels, Belgium) and the mean relative increases in skin thickness were determined.

Vaccination

The vaccine preparation (500 µL) contained 50 µg of SC mixed with 25 µg of MPLA (InvivoGen, San Diego, CA, USA) adjuvant.

Guinea pigs were randomly allocated to one of four groups. Group 1 guinea pigs (n=6) were vaccinated with the SC adjuvanted with MPLA. Group 2 guinea pigs (n=6) was the negative control group and received a preparation (500 µL) containing 25 µg of MPLA diluted in liquid Sab. Two additional control groups (groups 3 and 4), each consisting of three guinea pigs, received 500 µL of PBS only. Animals were vaccinated subcutaneously three times at two-week intervals (on days −70, −56 and −42) before challenge infection.

Challenge infection

On day 0 (42 days from the last vaccination) guinea pigs from groups 1, 2, and 3 were experimentally infected under general anaesthesia [medetomidine (500 µg/kg) and ketamine (40 mg/kg)] with *M. canis* while the animals from group 4 remained uninfected and served as a negative control of infection. The skin on the dorsum was shaved, gently abraded with a 25 G needle, and 250-µL inoculum containing 3×10^5 *M. canis* arthroconidia suspended in 5% (w/w) poloxamer 407 was applied to 15-cm^2 area of skin. Guinea pigs from group 4 were inoculated with 250 µL of poloxamer only.

Clinical and mycological follow-up

Infection sites were monitored weekly and evaluated clinically using four criteria: alopecia, erythema, scaling and crusts. The same investigator scored each animal and was blinded to the treatment groups. Each clinical criterion was evaluated on a scale of 0 to 3. Infection sites were examined with a Wood's lamp and given a score of 0 (no fluorescence on hairs) or 1 (positive fluorescence). *M canis* infections were confirmed by microscopic examination of

fluorescent hairs. A global score was calculated for each guinea pig by adding the clinical and fluorescence scores. Finally, a mean global score was calculated for each group.

Histology

At day 17 post-infection (PI), biopsy specimens were collected under general anaesthesia from one randomly selected guinea pig in each group. The selected animal showed clinical signs consistent with dermatophytosis. Samples were fixed in 10% neutralised buffered formalin and paraffin embedded for routine processing. To assess the invasion of keratinized skin structures by *M. canis*, 4-µm thick sections were stained with periodic acid-Schiff. The histopathological lesions were assessed using a routine haematoxylin-eosin staining.

Antibody response

In groups 1 and 2, blood samples (250 µL) were collected from the saphenous vein on days −70, −56, −42 and at two week intervals from day 0 to day 56 PI. The serum samples were obtained by centrifugation and stored at −20 °C until used.

An enzyme-linked immunosorbent assay (ELISA) was performed in the vaccinated and control groups. All assays were performed in one batch at the end of the study period. The antigens consisting of the *M. canis* SC, positive and negative reference antisera and rabbit anti-guinea pig immunoglobulins (Ig) were appropriately diluted after standard checkerboard titration. 96-well ELISA microplates (MICROLON® 600 High binding, Greiner Bio-One, Frickenhausen, Germany) were coated with 100 µL per well of 2.5 µg/mL SC diluted in PBS (pH 7.2) and incubated for 1 h at 37 °C. Odd-numbered rows were sensitized with the antigens while even-numbered rows remained free of antigen (control wells). After washing with PBS, unoccupied protein-binding sites were blocked by the addition of 200 µL per well of dilution buffer consisting of a 3.6% solution of casein hydrolysate (Merck, Whitehouse

166 Station, NJ, USA) in PBS containing 0.1% Tween 20 (PBS-T) for 1 h at 37 °C. Triplicate
167 serum samples were diluted 1:100 in the dilution buffer and 100 µL of each was added for 1 h
168 at 37 °C to both the antigens-coated and control wells. After washing four times with PBS-T,
169 100 µL of horseradish-peroxidase-conjugated rabbit anti-guinea pig Ig (Polyclonal Anti-
170 Guinea Pig Immunoglobulins/HRP, Dako, Glostrup, Denmark) diluted 1:1000 in dilution
171 buffer was added to each well. After a further-1-h incubation period at 37 °C and three
172 subsequent washes with PBS-T, peroxidase activity was revealed by addition of 100 µL of a
173 solution containing tetramethylbenzidine and hydrogen peroxide. The reaction was stopped
174 after 5 min by adding 100 µL of 1 N phosphoric acid and the absorbance at 450 nm was
175 measured directly with a Multiscan RC spectrophotometer (Thermo Labsystems, Vantaa,
176 Finland). On each ELISA plate positive and negative references were processed alongside the
177 samples in triplicate. The negative reference was serum from a guinea pig prior to *M. canis*
178 experimental infection (Mignon et al., 1999) and the positive reference was serum from the
179 same guinea pig collected 14 days after infection. Optical density was defined as the
180 difference between the mean absorbance for each triplicate serum sample tested and the
181 control wells.
182
183 **Quantification of IFNγ and IL-4 mRNA levels**
184 On day 56 PI, three guinea pigs from groups 1, 2, 3 and 4 were anaesthetised and blood
185 samples were collected by intracardiac puncture before euthanasia. Heparinised blood was
186 diluted 1:4 in calcium- and magnesium-free Hank's buffered salt solution (CMF-HBSS) and
187 incubated for 30 min at room temperature. Four mL of diluted blood was layered over 10 mL
188 of Ficoll-Paque™ PLUS 1077 solution (Amersham Biosciences, Uppsala, Sweden) and
189 centrifuged for 30 min at 400 × *g*. The peripheral blood mononuclear cell (PBMC) ring was
190 harvested, washed twice in CMF-HBSS and suspended in 24-well cell culture plates (Greiner

Bio-One) at a concentration of 5×10^5 cells/mL in RPMI 1640 + GlutaMAX™ medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% foetal calf serum (Gibco Life Technologies) and 1% penicillin-streptomycin (Gibco Life Technologies). Cells were stimulated with 5 µg of SC or with liquid Sab medium as a negative control for 72 h at 37 °C in a humidified atmosphere containing 5% CO_2. After stimulation, the PBMCs were collected by centrifugation and cell pellets were stored at −80 °C until use. All experiments were performed in triplicate.

Cell pellets were thawed on ice and total RNA was isolated using TRIzol® reagent (Invitrogen, Burlington, ON, Canada) as recommended by the manufacturer. The purified RNA was treated with DNase I (Invitrogen). Template cDNA was synthesized from RNA by reverse transcription using iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). Reverse transcriptase was omitted in the control reactions. The sequences of oligonucleotide primers for guinea pig 18S rRNA (internal control), IFNγ and IL-4 have already been published (Allen and McMurray, 2003; Oh et al., 2008) and were synthesized by Eurogentec (Liège, Belgium) (Table 1). The quantitative PCR reactions were assembled using the iQ™ SYBR® Green Supermix (Bio-Rad) and subjected to the following protocol in a MiniOpticon System (Bio-Rad): 10 min at 95 °C and 45 cycles of 45 s at 95 °C, 45 s at 60 °C and 45 s at 72 °C. The melting curve was performed from 45 °C to 95 °C in 1 °C/15 s increments. Results in terms of cycle thresholds were converted to folds 18S rRNA expression using the $2^{-\Delta\Delta Ct}$ method. The levels of cytokine mRNA in stimulated PBMCs were expressed relative to that in the negative control PBMCs.

Statistical analysis

The two-way analysis of variance (ANOVA) test followed by Bonferroni post hoc tests was used for the statistical comparison of both mean global scores and ELISA results between the

vaccinated and the non-vaccinated groups. It was also used for statistical comparison of DTH results. This test was performed with the GraphPad Prism 5.0 statistical software (GraphPad Software, San Diego, CA, USA). The levels of cytokine mRNA in stimulated PBMCs were compared with those determined in negative control PBMCs using a general linear model (GLM procedure of SAS; SAS Institute Inc., Cary, NC, USA). A *P* value of < 0.05 was considered as statistically significant.

RESULTS

Efficacy of the vaccine to prevent skin lesions development after *M. canis* challenge

The *M. canis* SC inducing the production of pro-inflammatory cytokines in feline PMNs (Cambier et al., 2013) represent attractive antigens to test in a vaccine study. These components were tested for their ability to elicit DTH responses in immune guinea pigs, i.e. having spontaneously recovered from an experimental infection with *M. canis*. Animals injected with the SC developed a significant increase in skin thickness 24 h after injection (Fig. 2). A vaccine study was therefore performed in guinea pigs with the *M. canis* SC adjuvanted with the MPLA, and a clinical and mycological follow-up was realised.

All three guinea pigs from group 3 (PBS), four of six guinea pigs from group 1 (SC + MPLA) and one of six guinea pigs from group 2 (MPLA) developed clinical signs consistent with dermatophytosis after *M. canis* challenge (Fig. 3). Typical skin lesions were observed at day 7 PI and were associated with a positive Wood's lamp and positive direct examination. Mean global scores accounting for clinical and fluorescence evaluations are shown in Fig. 4. Groups 1 and 2 had a significantly lower mean global score than group 3 on days 14 and 28 PI and on days 14, 21 and 28 PI, respectively. No significant difference was observed between groups 1 and 2. The application of poloxamer 407 without fungus did not produce any lesions in guinea pigs from group 4 (PBS, no challenge). Unlike clinical and mycological scores, histological

analyses performed on day 17 PI showed no obvious difference regarding to invasion of keratinized skin structures by *M. canis* between animals having received the MPLA (groups 1 and 2) and PBS-inoculated animals (group 3) (Fig. 5a). Subjectively, inflammatory lesions did not differ from one group to another (Fig. 5b, c).

Immune response conferred by the *M. canis* SC

Both the antibody response and the expression of IFNγ and IL-4 mRNA by PBMCs were evaluated along or at the end of the study period respectively.

Guinea pigs from group 1 (SC + MPLA) developed a high and rapid antibody response towards the *M. canis* SC (Fig. 6). After challenge infection, this antibody response still continued to increase and was maximal at the end of the experiment (day 56 PI). From day −42 until the end of the study, the antibody levels in guinea pigs from group 1 were significantly higher than those observed in animals from group 2 that received the MPLA adjuvant without the *M. canis* SC.

The level of IFNγ mRNA was higher in group 1 than in other groups (Fig. 7). However, a statistically significant difference was only observed by comparing with group 3. The level of IL-4 mRNA increased significantly in group 1 with regard to the group 2. However, this result was not interpretable as the level of this cytokine was also increased in group 4 (non-infected animals) by comparing with groups 2 and 3.

DISCUSSION

The most remarkable and unexpected result of this vaccination study was the partial protective effect conferred by the MPLA adjuvant in guinea pigs exposed to a challenge infection with *M. canis*. MPLA is a detoxified form of the endotoxin lipopolysaccharide recognised by TLR-4 (Johnson et al., 1987) and is used as a vaccine adjuvant in humans

(Thoelen et al., 1998). The MPLA improves the innate immune response to bacterial infections by increasing the number of cells with phagocytic functions at the sites of infection, which in turn enhances the bacterial clearance (Romero et al., 2011). This adjuvant is also able to stimulate the adaptive immune response by promoting the differentiation of CD4+ T cells into IFNγ-producing Th1 cells in mice (Thompson et al., 2005). In dermatophytosis, the protective immune response is considered to be of the Th1 type and associated with a DTH (Almeida, 2008; Mignon et al., 2008). However, in this study, no significant increase in IFNγ production was observed in guinea pigs having received MPLA alone suggesting that the partial protection conferred by the adjuvant was not related to its capacity to stimulate the adaptive immune system. Recent studies have demonstrated that the innate immune system has adaptive characteristics and could provide protection against infections in a B-/T-cell-independent manner (Bowdish et al., 2007; Netea et al., 2011; Quintin et al., 2012). In our study, MPLA could have triggered a non-specific innate immune response inducing protection in guinea pigs infected with *M. canis*. More precisely, this adjuvant could stimulate the cells of the innate immunity such as macrophages or polymorphonuclear neutrophils, allowing the elimination of the fungus.

Although the SC produce DTH in immune guinea pigs, they did not confer an additional protection effect with regard to the MPLA alone, suggesting that these fungal components could be not protective *per se*. The possibility that some particular proteins from the SC of *M. canis* could be useful as specific immunogens cannot be excluded. Indeed, the *M. canis* SC consist of a mix of proteins, some of which being potentially able to negatively modulate the immune response. Such immunomodulatory effects have already been revealed for other dermatophytes, such as *Trichophyton rubrum* able to grow inside macrophages after phagocytosis (Campos et al., 2006). Therefore, the characterisation and the selection of

appropriate antigens from the SC appear to be of major importance in the development of a highly effective vaccine against *M. canis*.

Vaccination induced a strong antibody response towards the SC and the production of IFNγ by PBMCs. Specific antibodies have been shown to be produced during *M. canis* (Sparkes et al., 1993) and other dermatophytic infections (Woodfolk et al., 1996) but no correlation has been observed between antibody levels and recovery from the disease. The strong antibody response induced by the *M. canis* SC could be irrelevant or even detrimental with regard to protection against challenge infection. The production of IFNγ in vaccinated animals is correlated with DTH reactions in immune guinea pigs and strengthens our hypothesis that the SC may contain Th1 antigens which are attractive candidates for further vaccination assays.

In conclusion, our results show that the MPLA adjuvant can induce *per se* a partial protection against a *M. canis* infection in guinea pigs while the crude fungal SC do not confer protection in our experimental conditions.

CONFLICT OF INTESREST STATEMENT

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the manuscript.

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Figure legends

Figure 1: Protein profile of the *Microsporum canis* secreted components (SC) separated in 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Lane A, SC; lane B, Sabouraud medium; lane C, protein marker. The molecular weights (in kDa) are indicated on the right.

Figure 2: Secreted components (SC) from *Microsporum canis* induce a strong delayed type hypersensitivity (DTH) reaction in guinea pigs having spontaneously recovered from an experimental infection. Typical DTH reactions were observed 24 h after intradermal injection of SC (2) in comparison with positive control (3) and Sab (1) used as negative control, in animals 50 days after experimental infection (b). The same antigens do not elicit obvious DTH reactions in naive (non-infected) guinea pigs (a). The increase in skin thickness was measured 24 h after injection and corresponds to the difference between values determined 24 h after and before injection. Data are representative of two experiments (mean ± SEM) and are expressed in percentage (c). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Figure 3: Comparative clinical follow-up of skin lesion development in vaccinated and control (PBS) guinea pigs after experimental infection with *Microsporum canis*. Pictures were taken at different stages of infection. They show skin lesions from 3 guinea pigs per group. In both group 1 (secreted components + MPLA) and 2 (MPLA), 3 out of 6 animals were randomly selected for pictures. Skin lesions were subjectively more severe in guinea pigs inoculated with PBS (group 3) than in those having received MPLA especially at days 14 and 28 after experimental infection. D, day post-infection are indicated on the left.

Figure 4: Kinetics of mean global score (± SEM) of infection in vaccinated and non-vaccinated guinea pigs after experimental infection with *Microsporum canis*. A significantly lower mean global score was observed in group 1 (secreted components + MPLA; n=6) and group 2 (MPLA; n=6) with regard to the group 3 (PBS; n=3) on days 14 and 28 post-infection (PI) and on days 14, 21 and 28 PI, respectively. No significant difference was observed between group 1 and group 2 throughout the study. Mean global scores of infection were assessed blindly on the basis of clinical and mycological criteria. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Figure 5: Evaluation of invasion of keratinized skin structures (a) and histopathological lesions (b, c) 17 days after experimental infection with *Microsporum canis*. Periodic acid-Schiff staining (a) reveals the presence of hyphae inside the hair shaft (long arrow) and the surrounding dermatophytic conidia (short arrow). Haematoxylin-eosin staining reveals significant inflammatory lesions (b) characterised by epidermal acanthosis (double arrow), erosions (*) and a moderate cellular infiltration in the dermis (†). Higher magnification shows epidermal spongiosis (c). The figure shows histological sections performed in a guinea pig from group 2 (MPLA). Scale bars represent 50 μm.

Figure 6: Evolution of the antibody response (optical density ± SEM) assessed by enzyme-linked immunosorbent assay (ELISA) against the *Microsporum canis* secreted components in guinea pigs from group 1 (secreted components + MPLA; n=6) and group 2 (MPLA; n=6). V: vaccination; C: challenge infection. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

Figure 7: Quantification of IFNγ and IL-4 mRNA levels in peripheral blood mononuclear cells (PBMCs) stimulated with the *Microsporum canis* secreted components (SC) in guinea

468 pigs from group 1 (SC + MPLA; n=6), group 2 (MPLA; n=6), group 3 (PBS; n=3) and group
469 4 [PBS (no challenge); n=3] at day 56 post-infection. The levels of IFNγ and IL-4 mRNA in
470 stimulated PBMCs were quantified by quantitative RT-PCR and expressed relative to that in
471 negative control PBMCs. Data are representative of three independent experiments (mean ±
472 SEM). *$P < 0.05$; **$P < 0.01$.