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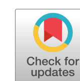
SHORT COMMUNICATION

Response with T_H1 profile obtained in vaccine formulation against Caseous Lymphadenitis in animal model C57 Black/6



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Abstract Caseous Lymphadenitis (CLA) is a contagious disease that compromises the quality of life of sheep and goats. Caused by *Corynebacterium pseudotuberculosis* CLA is responsible for considerable economic losses in sheep and goat farming. Therefore, the search for preventive measures, such as the development of vaccines is increasing. To evaluate the immunoprotective response of experimental vaccines different murine models susceptible to infections are used. Thus, the objective of this study was to evaluate the protective potential of the recombinant subunit vaccine using an endoglycosidase (rCP40) of *C. pseudotuberculosis* associated with Saponin and Complete Freund adjuvant (CFA) in murine model C57/Black6. Thus, four groups of animals were separated, where G1 and G2 were control groups and G3 and G4 were experimental groups (rCP40 + Saponin) and (rCP40 + CFA) respectively. The evaluation of the production of reactive antibodies to rCP40 showed that the animals inoculated with the adjuvants presented potentiation of the cellular and humoral immune response, presenting higher production of IgG2a and IgG2b. After the challenge, only the control groups died, while in the experimental groups, although some survived, they presented granulomas, which are characteristics of CLA.

Introduction

Caseous Lymphadenitis (CLA) is an infectious, chronic and subclinical disease that mainly affects small ruminants. It

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is characterized by the formation of abscesses that affect the world of sheep and goat farming (D'Afonseca et al., 2008; Dorella et al., 2006; Hoelzle et al., 2013; Williamson, 2001). It is caused by *Corynebacterium pseudotuberculosis*, a gram-positive, intracellular pathogen that is viable in the environment for long periods, which contributes to the spreading of CLA throughout the herd (Baird, 2007; Fontaine et al., 2006). *Corynebacterium* belongs to the group of *Actinomycetes*, which also includes the genera *Mycobacterium*, *Nocardia* and *Rhodococcus*. Both groups present common characteristics of the organization of the cell wall to a high level of guanine and cytosine in their genome (Dorella et al., 2006; Stackebrandt et al. 1997).

This disease is found worldwide and presents significant economic losses in the goat and sheep industry, which leads to decreased meat and milk production, skin depreciation and carcass condemnation (Paule et al., 2003; Pepin, Boisrame, & Marly, 1989). Brazil ranks 18th and 22nd among sheep and goat farmers in the world, respectively. They have a herd of about 26 million head, with 34% of goats and 66% of sheep and only in the Northeast region approximately 17 million animals are concentrated (IBGE, 2013). In this area, it is estimated that the disease prevails in herds of approximately 30% (Ribeiro, Dias Junior, Paes, Barbosa, & Nardi Junior, 2001).

The identification and elimination of infected animals are performed to prevent the spread of CLA by the herd. However, the most appropriate measure to contain this disease is vaccination (Paton, Walker, & Rose, 2003). There are currently available vaccines, although they are not licensed for use in many countries (Ruiz et al., 2011) and not all vaccines that have their permitted use for goats have the same efficacy in sheep. Nevertheless they can be seen as an efficient method for disease reduction through prevention once it reduces the number of contaminated animals in the herd, although staying away from eradication (Windsor & Bush, 2016).

Important targets such as the 40 kDa serine protease from *C. pseudotuberculosis* were identified when conferring protection on sheep in a study by Walker, Jackson, Eggleton, Meeusen, and Wilson (1994) and because of its proteolytic capacity was thus classified (Almeida et al., 2016). However, new characterizations have been made and the same is currently classified as an endoglycosidase (Shadnezhad, Naegeli, & Collin, 2016). Sequencing of a *C. pseudotuberculosis* strain FRC41 isolated from a 12-year-old girl inguinal lymph node with necrotizing lymphadenitis demonstrated that CP40 together with PLD were virulence factors encoded in the genome of this isolate (Trost et al., 2010). The immune response of recombinant CP40 in mice infected with *C. pseudotuberculosis* has recently been evaluated, and a greater induction of the immune response in murine was observed when compared to the response elicited by commercial vaccines (Droppa-Almeida et al., 2016; Silva et al., 2014).

To verify the immunoprotective potential of experimental vaccines, murine models susceptible to infections are used. In order to verify the parasite−host relationship during the infection with wild-type and attenuated strain of *C. pseudotuberculosis*, Fraga, 2012 used several murine models to verify the resistance to infection by the C57/Black 6 isogenic lineage, since it was not observed death or morbidity in any animal of this strain. Thus, it is interesting to use a murine model that presents a resistance to disease to verify the behavior of the vaccine in distinct hosts, once *C. pseudotuberculosis* presents a large host spectrum. However, there is little information about the antibodies response of C57/Black 6 mice to CLA. Therefore, the present study aims to evaluate the protective potential of the recombinant subunit vaccine using the endoglycosidase (rCP40) of *C. pseudotuberculosis* associated with different adjuvants in the C57/Black 6 murine model.

Material and methods

Ethics committee

This study was carried out in strict accordance with the recommendations of the Guide to Care and Use of Laboratory Animals governed by the National Council for the Control of Animal Experimentation (CONCEA). The Animal Research and Ethics Committee of Tiradentes University approved the protocol (Number: 010413). All efforts were made to minimize suffering.

Bacterial strains, growth conditions and plasmids

The plasmid used in the study was pAE/CP40 (Droppa-Almeida et al., 2016) and cultures of *E. coli* BL21 (DE3) Star were grown in Luria-Bertani (LB) medium or LB-agar at 37 °C for 18 h. When necessary, a total of 100 μg/mL ampicillin was added. The virulent strain of *C. pseudotuberculosis* used was the VD57 donated by the Federal University of Bahia. Its growth was done in Brain Heart Infusion (BHI) medium and 5% sheep blood Agar at 37 °C for 72 h.

Expression

Plasmid pAE/cp40 was inserted by heat shock on *E. Coli* BL21 (DE3) star. Expression was induced with the addition of 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) in LB medium and held in Shaker at 200 rpm at 37 °C for 3 h. Purification of the proteins was done by nickel affinity chromatography through the HisTrap™ column (GE). Confirmation of the purification was done by SDS-PAGE and the concentration of the protein by BCA kit (PIERCE).

Animals

Females of the C57Black/6 lineage aged 6–8 weeks were used for the vaccine potency test. The animals were obtained from the Gonçalo Moniz Research Center (CPqGM-FIOCRUZ-Salvador-Bahia). Also the animals were kept in the laboratory of the Institute of Health Sciences of the Federal University of Bahia (UFBA) where they had free access to water and a diet ad libitum in light/dark cycles of 12 h, at room temperature of 21 ± 2 °C.

Immunization and challenge

The animals were divided into four groups: 2 control groups (n = 5 each), Saponin Control Group (G1) and the Control Group Freund (G2) and 2 experimental groups (n = 10 each),

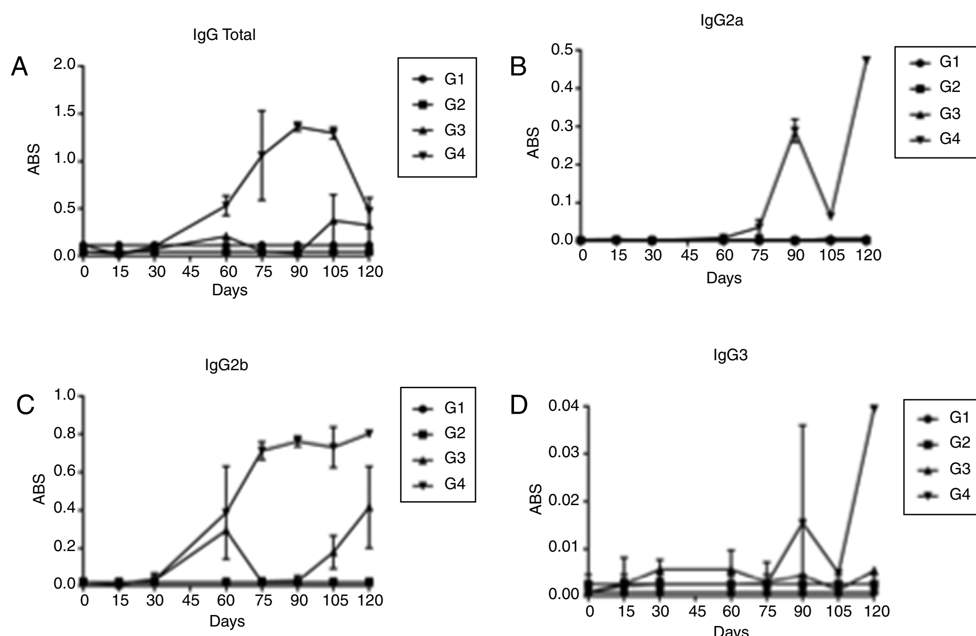

Figure 1 Post-immunization and post-challenge indirect ELISA. Determination of antibody levels of whole IgG and the subclasses IgG2a, IgG2b, IgG3 reactive to rCP40 at days 0, 15, 30 and 60 (post-immunization) and 0, 15, 30 and 60 post-challenge. A, whole IgG; B, IgG2a; C, IgG2b; D, IgG3. Control results are the mean of 5 animals in each group. *Means $P < 0.05$ between experimental groups (G4 and G4 groups) compared to the respective control group (G1 and G2 groups).

Experimental Group Saponin (G3) and The Freund Experimental Group (G4). Immunization was performed in three doses (15 day interval) of rCP40 (5 μg/mL) added with each of the adjuvants (Saponin and Complete Freund adjuvant [SIGMA]) and the route of administration used was the subcutaneous route. After 40 days of the last immunization the animals were challenged with (10^4 CFU) *C. pseudotuberculosis* VD57 by the intraperitoneal route and they were observed for 60 days to verify the presence of skin lesions or other clinical symptoms of CLA such as the formation of granulomas, loss weight, morbidity and mortality. For the serological tests blood samples were obtained on days 0, 15, 30 and 60 after the first immunization and on day 0, 30 and 60 post challenge. Serum from the animals was maintained at −20 °C.

Evolution of the immune response

The antibody production for total IgG or IgG1, IgG2a, IgG2b and IgG3 subclasses, reactive to rCP40 was performed by indirect ELISA using a protocol standardized by Oliveira, Langenegger, Langenegger, and Meyer (1992) as shown by Dantas (2004).

For the ELISA Immunoassay high-throughput 96-well plates (PERKIN ELMER) were sensitized with 50 μg of rCP40 per well in Buffered Carbonate Buffer (0.05 M, pH 9.6) and incubated overnight in a cold room at 4 °C. After three washes with phosphate buffered saline containing 0.05% Tween-20 (PBS-T20) the plates were blocked with 5% milk protein diluted in PBS-T20 for 2 h at 37 °C. Then the plates were incubated with the sera of the immunized animals at the concentration of 1:50 for 1 h at 37 °C. After incubation the plate was washed again and the addition of the anti-mouse conjugate (total IgG, IgG1, IgG2a, IgG2b and IgG3) at the 1:10,000 dilutions was done, after which it was incubated for 1 h at 37 °C. After washing, TMB (Bio-Rad) developer solution was added for 15 min at room temperature in the absence of light. The reaction was quenched with the addition of a 4 N solution of H_2SO_4. Optical density reading was performed in an automatic photocolorimeter for ELISA (Biorad, USA), with a 450 nm filter.

Statistical analysis

The GraphPad Prism version 6.0 for windows program (GraphPad Software, San Diego, CA) was used. To verify the homogeneity of the data, the Kolmogorov–Smirnov test was used, which resulted in non-parametric data. Therefore, the tests used were KrusKal–Wallis (Fisher, 1993) and the Wilcoxon *U* test – Mann–Whitney (Callegari-Jacques et al., 2003), where value of $P < 0.05$ was considered statistically significant. Fisher's test was used for the mortality and survival curve.

Results and discussion

The ability of the rCP40 protein to generate an immune response in the animals was assessed by progression of total IgG antibodies and subclasses IgG2a, IgG2b and IgG3 (Fig. 1) generated within the first 60 days post-immunization. In Fig. 1, G4 presented statistical differences at day 60 in total IgG production and for IgG2b and IgG3 in G3. For the other G1 and G2 groups, the levels of antibodies did not present statistical differences ($P > 0.05$).

After the challenge, the animals presented oscillations in the total IgG production during the evolution of the disease,

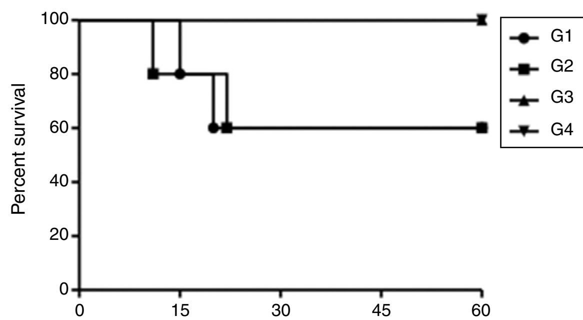

Figure 2 Percent survival of animals immunized with different vaccine formulations and challenged with *C. pseudotuberculosis* strain VD57 (10^4 CFU). Regarding to the groups G1 and G2 which presented an $n = 5$ and the groups G3 and G4 presented an $n = 10$ animals.

but a gradual increase was observed during the analysis, where day 30 ($P < 0.05$) represents the point that presented the highest production of these specific antibodies. Although the groups inoculated with Freund's adjuvant had higher levels of antibody production, they did not present statistical differences ($P > 0.05$) when compared to the group inoculated with Saponin. From days 30 and 60 there was a significant increase in the production of these antibodies to be compared with the control groups ($P < 0.05$). After challenge the production of specific IgG3 antibodies becomes significant in the G3 group, which indicates a T_H2 type response. G4 did not present statistical differences for IgG3, presenting a T_H1 profile. The reactivity and production of specific isotypes for IgG2a, IgG2b and IgG3 rCP40 is associated with the performance of proinflammatory cytokines (Hartmann, Eschbach, & Breloer, 2012; Zhang, Goldschmidt, & Salter, 2012), production of IFN-α and helper $CD4^+$ T cells, which activated B cell activation by modifying the immunoglobulin heavy chain (Mohr et al., 2010). Therefore we verified the joint action of the cellular and humoral immune response generated by the recombinant protein rCP40.

After the challenge the animals were observed for 60 days and with the data from the observations a survival curve was constructed (Fig. 2). Both G1 and G2 control groups had 2 deaths, whereas the experimental groups did not present any deaths. However, according to the Fisher test there were no statistical differences between the groups, presenting a *P* value of 0.154. Facing the absence of death, the formation of granulomas was an important point to be evaluated. In group G3 in the same period presented 2 animals with granulomas and in the G4 3 animals with granulomas, both with an $n = 10$. Even with the considerable production of specific antibodies for rCP40, it was not possible to obtain full protection of the post-challenge animals, since they presented granulomas, a characteristic sign of CLA. The susceptibility of Balb/c and C57/Black 6 mice to experimental infection with CLA has been studied. The results showed that these animals are differently susceptible to the infection (Fraga, 2012). Most of the investigations about host antibodies response to CLAS have been carried out on Balb/c mice, so in this work antibodies response of C57 black mice to this infection was studied.

Conclusion

The results presented in this paper confirm the higher resistance against CLA against the C57 Black/6 strain, where in previous studies by Droppa-Almeida et al. (2016) used the Balb/C line and the control groups presented death due to the use of the virulent strain VD57, the same as used in this work. G4, although presenting higher specific immunoglobulin levels for the vaccine, was the experimental group that presented more formation of granulomas, identifying the need for a more effective cellular response for the protection of the animals against CLA. In view of this, new studies on the diversity of murine models and their susceptibility to CLA are necessary, given the lack of studies in the area.

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Conflicts of interest

The authors declare no conflicts of interest.

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