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Specific and cross-reactive immune response against *Mycobacterium tuberculosis* antigens in mice immunized with proteoliposomes from *Mycobacterium bovis* BCG

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ABSTRACT

Objective: To characterize the immunogenicity and the induction of cross-reactive responses against *Mycobacterium tuberculosis* (*M. tuberculosis*) of a proteoliposome (PL) from *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) with and without alum hydroxide (AL) as adjuvant (PLBCG-AL and PLBCG, respectively) in BALB/c mice.
Methods: BALB/c mice were inoculated with phosphate buffer solution, BCG, PLBCG and PLBCG-AL. The humoral immunogenicity was determined by ELISA [immunoglobulin G (IgG), IgG1 and IgG2a] and the cellular immunogenicity was evaluated *in vivo* by delayed type hypersensitivity. The humoral cross-reactive response against *M. tuberculosis* was determined by Western blot.
Results: Sera from animals immunized with PLBCG-AL and PLBCG showed significant increase in specific total IgG and IgG1 antibodies and the presence of cross-reactive antibodies against *M. tuberculosis* antigens, which were more intense with the use of alum as adjuvant. Mice immunized with PLBCG and PLBCG-AL also showed a specific cellular response *in vivo*.
Conclusions: The cellular and humoral immunogenicity of PLBCG and the capacity to induce cross-reactive responses against *M. tuberculosis* is in agreement with the protective capacity previously demonstrated by this vaccine candidate and supports the continuation of its evaluation in further stages.

1. Introduction

Due to the controversial protective efficacy of the current tuberculosis vaccine, Bacillus Calmette–Guérin (BCG), intensive efforts are currently devoted to design improved vaccines [1–3]. Given the high degree of homology shared between *Mycobacterium tuberculosis* (*M. tuberculosis*), the causative agent of tuberculosis, and other mycobacteria, we explored the possibility of using proteoliposome (PL) prepared from BCG as an experimental vaccine against tuberculosis with encouraging results in a challenge model in mice [4].

PL or outer membrane vesicles (OMV) comprises detergent extracts of the outer membrane components of bacteria [5]. VA-MENGOC-BC™, the Cuban vaccine against *Neisseria meningitidis* serogroup B, is one of the few examples of licensed PL-based vaccines [6]. In the specific case of tuberculosis vaccine development, different formulations containing membrane and cell wall components, including natural OMV, have been tested in experimental animals demonstrating immunogenicity, protective and immunomodulatory capacity [4,7–10].

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All the procedures were approved by the Animal Experimentation Ethics Committee of the Finlay Institute and were carried out according to the international regulations of laboratory animal experimentation.

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In a previous study, some aspects of the humoral immunogenicity of PLBCG administered with incomplete Freund's adjuvant were explored, demonstrating the recognition of BCG antigens and epitopes as well as antigenic fractions of *M. tuberculosis* by animals immunized with this formulation [11].

Taking into consideration the induction of protection against tuberculosis after the immunization of mice with PLBCG and alum hydroxide (AL) (PLBCG-AL) [4], the main objective of this work was to characterize the immunogenicity and the capacity of PLBCG to induce cross-reactive responses against *M. tuberculosis* antigens in mice, using AL as adjuvant.

2. Materials and methods

2.1. Organism

BCG Moreau strain (Biologicals Production Enterprise, Carlos J. Finlay, Cuba) was used. The BCG biomass was obtained by conventional cultivation in Sauton medium [12].

2.2. PLBCG

PLBCG was produced according to the methodology described by Tirado *et al.* [4]. Briefly, BCG biomass was treated with sodium deoxycholate (5%–15%, w/v) (0.10–0.25 mL/g biomass) followed by a centrifugation (17 700 ×*g* for 20 min at 4 °C). The supernatant was ultra-centrifuged (65 000 ×*g* for 2–8 h at 4 °C) and the pellet was re-suspended, filtered through Sartorius™ Minisart™ plus syringe filters (0.2 μm) and stored at 4 °C.

2.3. Animals

Forty female BALB/c mice (6–8 weeks) from the National Center for the Production of Laboratory Animals, Cuba, were used for this study. All the procedures were approved by the Animal Experimentation Ethics Committee of the Finlay Institute and were carried out according to the international regulations of laboratory animal experimentation [13].

2.4. Immunization of mice

Mice were divided into four groups (n = 10) and were inoculated subcutaneously with 100 μL of the following inocula: phosphate buffer solution (PBS); BCG (10^6 CFU of BCG, single inoculation), PLBCG (25 μg of PLBCG) and PLBCG-AL [25 μg of PLBCG + 1 mg of AL (Alhydrogel, Sigma, Darmstadt, Germany), respectively]. Mice received two doses on Days 0 and 21; except for the BCG group, which received a single dose on Day 0. Blood samples were taken 42 days after the first immunization. These samples were centrifuged (7 000 ×*g* for 10 min) and sera was collected and stored at −20 °C.

2.5. Humoral immunogenicity

Specific anti-PLBCG immunoglobulin G (IgG), IgG1 and IgG2a in sera from mice were determined by ELISA as previously described [14], using the PLBCG as coating antigen at 20 μg/mL. The plates were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Sigma, Darmstadt, Germany) at a dilution of 1:1 000 or rabbit anti-mouse IgG1 and IgG2a (Sigma, Darmstadt, Germany) at a dilution of 1:8 000.

2.6. Delayed type hypersensitivity (DTH)

The ability to induce DTH in the immunized animals was evaluated 42 days after the first immunization. Mice received intradermally 100 μL of inocula (50 μg of PLBCG) in the dorsal region of the left footpad. PBS was administered at the contra lateral extremity as negative control. The diameter of induration produced was measured at 24, 48 and 72 h, using calipers with an accuracy of down to 0.05 mm. The size of induration in the immunized mice was estimated as the difference between the diameters of induration obtained in left and right footpads.

2.7. Cross-reactivity against M. tuberculosis antigens

The cross-reactivity was studied with pooled sera of animals immunized with PBS, BCG, PLBCG and PLBCG-AL against *M. tuberculosis* antigens [soluble cell wall proteins (SCWP) and CWP of *M. tuberculosis*, Colorado State University]. Samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (acrylamide 12.5%) and studied by Western blot according to standard procedures [15,16]. The nitrocellulose membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG (Sigma, Darmstadt, Germany).

2.8. Data management

The results of humoral and cellular immunogenicity determinations were analyzed as follows: an exploratory analysis of the data using box or box and whisker plots was carried out. The normality and homogeneity of variance were analyzed with Shapiro–Wilk normality and Levene tests respectively. One-way ANOVA and Tukey's range tests were applied for comparison between the groups. P values less than 0.05 were considered statistically significant.

3. Results

Analysis of the total anti-PLBCG IgG response showed that the immunization with PLBCG-AL elicited a higher antibody response compared to the other groups ($P < 0.001$) (Figure 1). BCG and PLBCG immunized groups showed a statistical increase in the specific IgG response compared to the group receiving PBS ($P < 0.001$) (Figure 1). BCG immunization

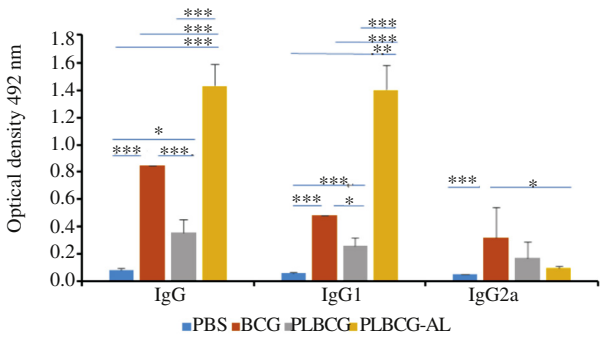

Figure 1. Humoral immunity induced by PLBCG.
Total IgG, IgG1 and IgG2a response against PLBCG of BALB/c mice (n = 5 per group) that received PBS, BCG, PLBCG or PLBCG-AL. Results are represented as mean ± SD. One way ANOVA and Tukey multiple comparison tests were used to analyze the data. *: $P < 0.05$, ***: $P < 0.001$.

induced significant increase of specific IgG compared to mice receiving PLBCG ($P < 0.001$).

The response of specific IgG1 showed a similar behavior than the specific IgG response (Figure 1). The level of specific IgG2a from BCG immunized mice showed significant differences compared to animals receiving PBS ($P < 0.001$) or PLBCG-AL ($P < 0.05$) (Figure 1).

In the DTH study the results showed that 24 and 48 h after immunization, there were no significant differences between the groups (Figure 2). However, a statistically significant increase in the induration was observed in groups immunized with BCG, PLBCG and PLBCG-AL compared to the group inoculated with PBS 72 h after inoculation (Figure 2).

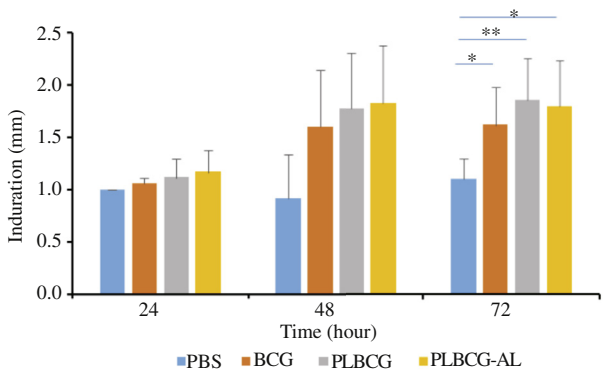

Figure 2. DTH response of BALB/c mice three weeks after the last immunization with PBS, BCG, PLBCG or PLBCG-AL.
The induration was measured at 24, 48 and 72 h. *: $P < 0.05$; **: $P < 0.01$.

The cross-reactivity of sera of mice immunized with either, PBS, BCG, PLBCG or PLBCG-AL against *M. tuberculosis* antigens (SCWP and CWP) was evaluated by Western blotting (Figure 3). Sera of animals inoculated with PBS did not display reactivity with any of the mycobacterial antigens used. Animals immunized with BCG showed cross-reactivity with SCWP (30 and 35 kDa bands) or CWP (35 and 45 kDa bands) of *M. tuberculosis* with discrete intensity. Sera from animals immunized with PLBCG-AL showed strong reactivity against a wide range of bands in SCWP (defined bands approximately at 38, 45 and 70–100 kDa) and CWP (several bands between 35 and 50 kDa) (Figure 3).

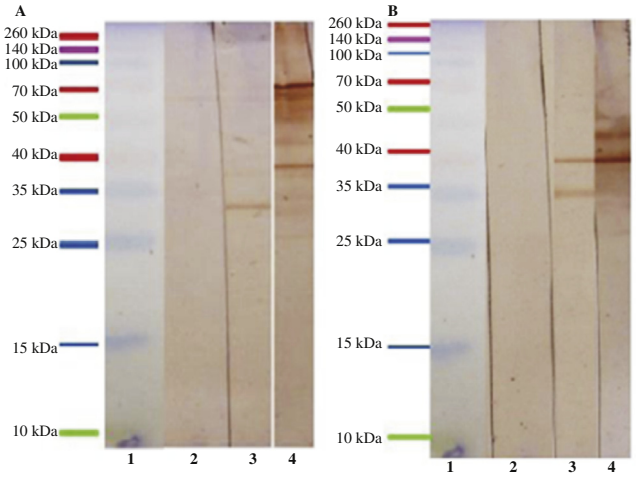

Figure 3. Cross-reactivity of sera of mice immunized with PLBCG against *M. tuberculosis* antigens.
A: SCWP of *M. tuberculosis*; B: CWP of *M. tuberculosis*. Lane 1: Molecular weight markers; Lane 2: Sera from mice inoculated with PBS; Lane 3: Sera from mice immunized with BCG; Lane 4: Sera from mice immunized with PLBCG-AL.

Animals immunized with PLBCG showed a very discrete recognition of few bands between 35 and 70 kDa in CWP (data not shown).

4. Discussion

PL contains lipopolysaccharides, proteins and other molecules in their structure known as pathogen associated molecular patterns with immunogenic potential and modulator effects [17–19]. The use of PL and their derivatives as vaccines and/or adjuvants is a subject of growing interest [4–6,17–20].

In the present study we evaluated the immunogenicity of a PLBCG which have previously been shown to elicit protective responses against *M. tuberculosis* in mice [4]. This experimental vaccine candidate induced equal protection to that of BCG when administered with AL and resulted in superior protection than BCG when administered as booster of BCG which supports its further evaluation as tuberculosis vaccine candidate [4].

Regarding the specific IgG response, it is interesting to note that although both PLBCG and PLBCG-AL showed statistical increases compared to non-immunized animals, the intensity of the response was higher with the use of AL, even superior to the response elicited by BCG.

The induction of antibodies against PLBCG components was not dependent on the administration of live bacteria which is an indication of the immunogenic potential of the formulation even without the addition of adjuvants.

Regarding the observed potentiating effect of the addition of AL in the specific humoral immune response, similar results were obtained in a previous study where the same adjuvant (AL) was used with PL of *Mycobacterium smegmatis* (PLMs) [14].

The increase in IgG1 reactivity without induction of specific IgG2a, by PLBCG, more prominent with the use of AL, observed in the subclasses study, suggest a T helper (Th)2 pattern of response which is reported to be associated with the use of AL as the adjuvant [21,22].

It is considered that Th2 immune responses are associated with a poor evolution and protection in tuberculosis [23], but a recent report raised the possibility that Th2 immune responses mediated by a T cell subset with cytolytic or regulatory capacity could be important for the induction of protection against tuberculosis [24].

However, previous studies performed with PLMs adsorbed with AL indicated that the formulation stimulated the generation of a mixed Th1/Th2 pattern [14] as in the case of the adjuvant AS04, which included a toll like receptor 4 agonist formulated with AL, which elicited a balanced Th1/Th2 response [25].

DTH evaluation showed a significant response with the use of PLBCG with and without AL, which is an indication that the intrinsic Th1 stimulatory capacity of PLBCG is not abrogated by the formulation with AL. This is an important result considering the relevance of Th1 responses in the protection against tuberculosis which could explain, in part, the protective effect of this formulation upon challenge with *M. tuberculosis* in mice [4]. Considering the predicted presence of proteins in PLBCG with identity with important cell wall proteins of *M. tuberculosis*, the induction of Th1 immune response against them associated with the protective capacity of PLBCG cannot be ruled out [11].

In a previous study, the immunization of mice with PLMs did not induce a DTH immune response against the PL but in contrast, induced cross-reactivity against antigens of *M. tuberculosis* [14].

It has been postulated that the formulation of PL with AL increase the stability of the formulation favoring the slow release of antigens without interference with the induction of Th1 responses [26].

The humoral cross-reactivity observed in the sera of animals immunized with PLBCG-AL against *M. tuberculosis* antigens is not surprising, considering the high antigenic and genetic homology between BCG and *M. tuberculosis* [27].

In fact, we have reported, after immunization with PLBCG with incomplete Freund's adjuvant the recognition of antigenic fractions of *M. tuberculosis* and shared B cell epitopes between BCG and *M. tuberculosis* belonging to surface proteins [11].

The humoral cross reactive response against *M. tuberculosis* antigens elicited by PLBCG-AL could contribute to the protective effect of this formulation in mice [4] considering the potential role of specific antibodies in the protection against *M. tuberculosis* [28–33]. Antibodies could modify the outcome of mycobacterial infection by different mechanisms such as toxin neutralization, opsonization, enhancement of antigen presentation, complement activation, interference with adhesion, increase in cytokine expression and enhancement of phagosome-lysosome fusion among others [28,29,31,33]. According to results obtained by us and other groups with monoclonal antibodies directed to surface antigens to *M. tuberculosis* which mediated protection against the infection, it had not been evident that the capsule hinders the access of the antibodies to their targets [34–38]. Natural outer membrane vesicles produced by *M. tuberculosis* and BCG has an immunomodulatory effect and contribute to the virulence of *M. tuberculosis* [39]. The immunogenicity of natural OMV from *M. tuberculosis* has been demonstrated to elicit a mixed Th1/Th2 pattern of stimulation as in the case of our formulation but without the use of adjuvants [39].

Natural OMV of *M. tuberculosis* induced protection against *M. tuberculosis* and a booster effect in animals immunized with BCG, but such effect was not produced after the immunization with natural OMV of BCG [39], which is in contrast with our results after the immunization with PLBCG with AL which conferred similar protection with BCG and better protection than BCG after their use as booster of BCG in mice [4]. This could be explained by a better presentation and/or the inclusion of more relevant antigens for protection in vesicles obtained by detergent extraction and formulated with AL as in the case of our vaccine candidate.

The induction of specific cellular and humoral immune responses in animals immunized with PLBCG-AL exhibiting cross-reactivity against *M. tuberculosis* antigens supports further evaluation of this vaccine candidate.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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