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Protective capacity of proteoliposomes from *Mycobacterium bovis* BCG in a mouse model of tuberculosis

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Abbreviations: TB, Tuberculosis; Mtb, *Mycobacterium tuberculosis*; PL Proteoliposomes; BCG, Bacillus Calmette-Guerin; PBS, Phosphate Buffer Solution; CFU, Colony Forming Unit; PLBCG, Proteoliposomes obtained from BCG; PLBCG-Al, PLBCG adjuvanted with alum; SD, standard deviation

Tuberculosis (TB) is one of the most important causes of mortality and morbidity due to infectious diseases. BCG, the vaccine in use, is not fully protective against TB. In a previous study, we have shown that proteoliposomes (outer membrane extracts), obtained from BCG (PLBCG) were able to induce humoral immune responses against *Mycobacterium tuberculosis* (Mtb) antigens. With the objective to evaluate the protective capability of PLBCG alone or as a booster with BCG, a murine model of progressive pulmonary TB was used. Animals immunized with PLBCG adjuvanted with alum (PLBCG-Al) showed similar protection to that conferred by BCG. The group immunized with PLBCG-Al as a booster to BCG gave superior protection than BCG as evidenced by a reduction of bacterial load in lungs 2 months after infection with Mtb. Animals immunized with BCG, PLBCG-Al and this formulation as a booster of BCG, showed a significant decrease of tissue damage (percentage of pneumonic area/lung) compared with non-immunized animals. These results demonstrate that immunization with PLBCG-Al alone or as a booster to BCG induce appropriate protection against challenge with Mtb in mice and support the future evaluation of PLBCG as a promising vaccine candidate against Mtb.

Introduction

Tuberculosis (TB) is one of the most important causes of mortality and morbidity due to infectious diseases.[1–5] Bacillus Calmette-Guerin (BCG) is the current vaccine approved for human use against TB.[1-5] It is most effective in protecting children from the severe forms of the disease; while its efficacy in adults is poor especially against pulmonary TB.[1-5] It has been suggested that boosting of the primary BCG vaccination may produce enhanced protection against TB.[4,5] Many studies support the role of mycobacterial cell wall components in the development of TB pathogenesis and therefore have been a prime target for the identification and characterization of antigens with potential application in vaccine development.[6-9] Proteoliposomes (PL) are detergent outer membrane extracts of bacteria that contain proteins, lipids and native lipopolysaccharides.[10] Only a few PL-based vaccines are licensed, one of them, VA-MENGOC-BC®, is a vaccine composed of PL obtained from the outer membrane of *Neisseria meningitidis* serogroup B.[11] This PL has been shown to have high efficacy in controlling meningococcal disease and exhibited adjuvant effect when used with other antigens.[11,12] BCG has high genome and antigenic homology to that of Mtb.[13-15] We reported that PLBCG induced humoral immune responses against Mtb antigens in mice.[16] In this study, we evaluated the protective effect of PLBCG in a murine model of intratracheal infection and its potential use as a booster to BCG vaccination.

Materials and Methods

Organism

BCG Moreau strain, (Enterprise of Production of Biologicals, Carlos J Finlay, La Habana, Cuba).

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PLBCG

The BCG culture was centrifuged at 17,700 × *g* for 30 min at 4°C. After two washes, the pellet was resuspended in buffer solution (30 mM Tris, 2 mM EDTA, pH 8.5) in the presence of sodium deoxycholate [5-15% (w/v)] (0.1-0.25 ml/g biomass). One hour later, the sample was centrifuged as above and the supernatant collected and ultracentrifuged at 50,000-70,000 ×*g* for 2-8 h at 4°C. Finally the pellet was resuspended in buffer solution, filtered (Sartorius Minisart-plus 0.45 and 0.2 μm filters) and stored at 4°C. Characterization was carried out as described.[16,17]

Animals

Sixteen male Balb/c mice (6-8 weeks) were used in this study. The animals were kept in special boxes coupled to a negative-pressure micro-isolator.

Challenge study

Protection induced by the PLBCG was evaluated against Mtb infection in mice. Mice were distributed in 4 groups (n = 4) and were inoculated subcutaneously with 100 μL of the following inocula: PBS, BCG (10^6 CFU of BCG, single inoculation), PLBCG-Al [50 μg PLBCG +1 mg Alum (Alhydrogel, Sigma), 2 doses were administered with an interval of 3-weeks], and BCG+PLBCG-Al (10^6 CFU of BCG and 3 weeks later 50 μg PLBCG-AL). Two months after the last inoculation, mice were challenged with 2.5 × 10^5 CFU of H37Rv Mtb strain in 100 μL of PBS by the intratracheal route as previously described.[18,19] All animals were euthanized 2 months after Mtb infection under anesthesia with pentobarbital.[18,19] All procedures were performed in a class III cabinet in a biosafety level III facility according to the guidelines [20] and approved by the Animal Experimentation Ethics Committee of the National Institute of Medical Sciences and Nutrition "Salvador Zubirán" (INCMNSZ) of Mexico.

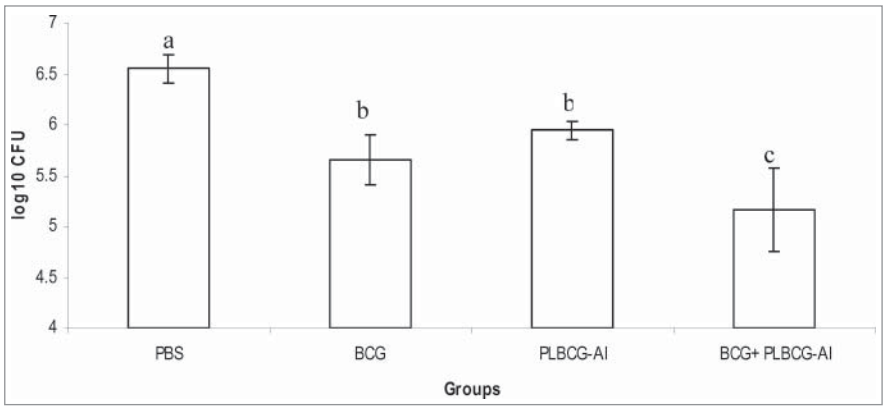

Figure 1. Determination of bacterial load in lungs of mice challenged with Mtb. Mice were challenged by intratracheal route 2 months after inoculation. Groups: PBS, BCG (10^6 CFU of BCG, one inoculation), PLBCG-Al [50 μg PLBCG +1 mg Alum (Alhydrogel, Sigma), 2 doses administered in a 3-week interval], and BCG+PLBCG-Al (10^6 CFU of BCG and 3 weeks later 50μg PLBCG-AL). The statistical analysis was performed by one-way ANOVA followed by Multiple Range test. Each bar represents the mean ± SD. Different letters denotes significant statistical difference between the groups. $P < 0.001$. (n=4 per group)

Bacilli load

The bacilli load was determined by CFU of Mtb in lung homogenates. Four lungs per group were disrupted separately in a Polytron homogenizer (Brinkmann Instruments, Rexleid, Canada) in 1 ml of isotonic salt solution containing 0.05% Tween 80 (Sigma), and 100 μL of this homogenate in fold10- serial dilutions were plated on Petri dishes containing Bacto Middlebrook 7H10 agar (Difco, USA) and incubated at 37°C. Colonies were counted twice under a stereoscopic microscope after 14 d of incubation.

Histopathology and morphometric studies

The right lung of 3 mice per group was perfused via the trachea with 100% ethanol and processed for histological studies. Sections were stained with haematoxylin and eosin.[19] The pneumonic areas were measured and analyzed using Leica Q-win system software (Leica Microsystems Imaging Solutions LTD, Cambridge, UK, 25x).

Data management

Measurements were made blind, and data of log $_{10}$CFU and percentage of pneumonic area/lung are expressed as the mean ± SD. One way ANOVA and Multiple Range test were used for the determination of significant differences.

Results and discussion

Mtb cell wall components have been suggested to contain virulence factors and protective antigens.[6-9,21] Taking into consideration the high antigenic and genetic homology between Mtb and BCG[13-15] and our previous results eliciting cross reactive immune responses after the immunization with PLBCG-Al,[16] it is reasonable to assume that PLBCG-Al can be a potential vaccine candidate against TB.

In this paper we evaluated the protective effect of PLBCG-Al in a mouse model of intratracheal infection and its potential to be used as a booster to BCG vaccination. The protective effect was measured by determining the bacterial load and the tissue damage (percentage of pneumonic area) in the lungs of immunized mice compared to PBS group and with animals receiving BCG.

Taking into consideration that the evaluation of protection was carried out 2 months after challenge and the fact that the mortality in the model used appears between 2 and 4 months post-challenge, the study of survival was not included in this experiment.

As shown in **Figure 1**, PLBCG-Al and BCG+PLBCG-Al groups were capable of significantly reducing the bacterial load in the lungs in comparison with non-treated

animals ($p < 0.001$). While PLBCG-Al immunized animals showed similar levels of CFU reduction as those of the BCG group, mice immunized with PLBCG-Al as a booster to BCG (BCG+PLBCG-Al) displayed significantly lesser CFU compared to BCG-vaccinated animals ($p < 0.001$).

Regarding the lung surface area affected by pneumonia, as shown in Figure 2, mice immunized with BCG, PLBCG-Al and BCG+PLBCG-Al showed significantly fewer percentage of lung pneumonic area than animals receiving PBS ($p < 0.001$). In contrast with the results obtained with the bacterial loads in lungs there are no statistical differences between the groups receiving PLBCG-Al as a booster of BCG compared with animal receiving PLBCG-Al or BCG respectively.

In general there was correspondence between CFU and lung surface area affected by pneumonia.

Similar results were obtained of bacterial load and pneumonic area in another experiment using animals immunized with BCG Phipps strain as a control group (data not shown).

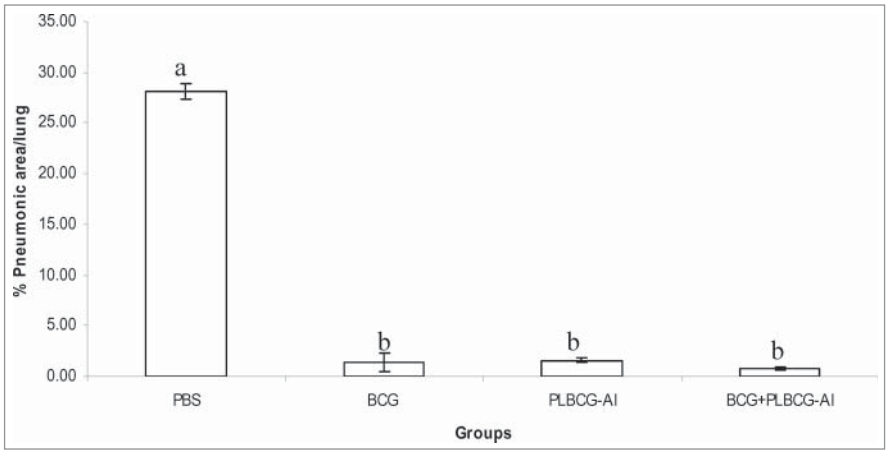

Figure 2. Determination of pneumonic area in lungs of mice challenged with Mtb. Mice were challenged by intratracheal route 2 months after inoculation. Groups: PBS, BCG (10^6 CFU of BCG, one inoculation), PLBCG-Al [50 μg PLBCG +1 mg Alum (Alhydrogel, Sigma), 2 doses were administered in a 3-week interval], and BCG+PLBCG-Al (10^6 CFU of BCG and 3 weeks later 50μg PLBCG-Al). Morphometric study was carried out with light microscopy using Leica Q-win System Software. Statistical analysis was performed by one-way ANOVA followed by Multiple Range test. Each bar represents the mean ± SD. Different letters denotes significant statistical difference between the groups. $p < 0.001$. ($n = 3$ per group).

The protection conferred by BCG could last for up to 20 y[1-5,22-30] The peak of incidence of TB increases progressively up to adulthood probably related with the lost of protection provided by BCG vaccination.[31]

Ethical issues related with the possibility to use control groups without BCG vaccination in clinical trials may difficult the evaluation of vaccines aimed to replace BCG, which is an intervention preventing at least the mortality and sequelae associated with the severe forms of TB in childhood.[31] Therefore it would be more acceptable that the first generation of new TB vaccines be based on candidates to be administered as boosters to the primary BCG vaccination. In this way the advantages of BCG vaccination are retained (protection against severe forms of the disease in childhood and efficacy window of 10-20 y for pulmonary TB as well as for leprosy) while re-enforcing protection with a heterologous vaccine to boost the initial protection afforded by BCG.[31] This concept is reflected in the current clinical trials where most of the candidates are evaluated as boosters to primary BCG vaccination.[31]

Despite of this, vaccine candidates aimed at replacing BCG as recombinant BCG strain VPM 1002, and live attenuated Mtb strain MTBVAC are in different phases of clinical evaluation.[32] Many other candidates with potential to replace BCG are in preclinical evaluation.[32]

A variety of subunit and live vaccines have been developed as booster vaccines.[31,32] Once successful results are obtained with them used alone, they are tested as booster of BCG. Therefore, it is notable that our formulation adjuvated with alum showed levels of protection comparable to that detected in BCG and can exceed BCG regarding bacterial loads in lungs when used as a booster to BCG.

Priming with BCG and booster with recombinant Ag85B induced superior protection against Mtb than BCG in guinea pigs.[33] In the same study, immunization with recombinant BCG over-expressing Ag85B gave superior protection than BCG; however, not further protection was obtained after booster with recombinant Ag85B in these animals.[33]

An Ag85A DNA vaccine used as booster to BCG did not enhance protection compared with BCG.[34] However, the DNA vaccine used as prime immunization followed by BCG booster improves the protection.[34]

Adenovirus-vectored vaccine expressing Ag85A (AdAg85A), used as intranasal booster, increased the protection conferred by BCG.[35]

In another study, recombinant modified vaccinia virus Ankara expressing antigen 85A (MVA85A) have shown to be highly immunogenic in humans.[36] However, although the BCG prime-MVA85A boosting regimen has successfully completed Phase I clinical trials, in Phase II b clinical trials in South Africa has failed to show significant protection against TB and Mtb infection.[36,37] However, this vaccine candidate could be protective in adolescents and adults which have a well-developed immune response and could be protective in all age groups against severe disease.[37]

Proteoliposomes of Mycobacterium smegmatis elicited humoral immune responses against mycobacterial lipids in mice, demonstrating the presence of lipid components in mycobacterial proteoliposomes.[17]

Lipds are important potential targets for vaccine development against TB considering the importance of these components in the virulence of Mtb.[37,39]

Several vaccine candidates containing mycobacterial lipids have demonstrated protective capacity in guinea pigs and mice[40-43]

The current study suggests that PLBCG-Al as a booster to BCG could be a new alternative candidate to be tested for protection against TB in further phases of evaluation.

One advantage of this strategy is that it not rely only on the booster with one or few antigens, but instead, it uses a candidate containing a variety of antigens, mainly associated with the cell wall, which boost a wide array of responses, probably both cellular and humoral, to promote a good overall level of protection. Another potential advantage is the possibility to be used in HIV-positive infants currently not receiving BCG vaccination due to the risk of disseminated BCG infection and mortality[44]

Conclusions

Our results demonstrated, for the first time, the protective effect of PL obtained from BCG adjuvanted with alum (PLBCG-Al) in a mouse model of intratracheal infection with Mtb. Furthermore, we have demonstrated that this PLBCG-Al could be used as a potential booster to BCG. The prime-boost vaccination with BCG+PLBCG-Al provided even greater protection than BCG alone. Therefore, the BCG+PLBCG-Al combination could be a potential vaccine strategy against TB which deserves further studies to evaluate its feasibility and efficacy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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