Tuberculosis 101 (2016) 44–48

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

IMMUNOLOGICAL ASPECTS

Mycobacterium smegmatis proteoliposome induce protection in a murine progressive pulmonary tuberculosis model

Yanely Tirado [a], Alina Puig [a], Nadine Alvarez [a], Reinier Borrero [a], Alicia Aguilar [a], Frank Camacho [a], Fatima Reyes [a], Sonsire Fernandez [a], Jose Luis Perez [a], Reynaldo Acevedo [a], Dulce Mata Espinoza [b], Jorge Alberto Barrios Payan [b], Maria de los A. Garcia [a], Ramlah Kadir [c], María E. Sarmiento [c], Rogelio Hernandez-Pando [b], Mohd-Nor Norazmi [c, d, **], Armando Acosta [d, *]

[a] Institute Finlay, La Habana, Cuba
[b] Experimental Pathology Section, Department of Pathology, National Institute of Medical Sciences and Nutrition "Salvador Zubiran", D.F. Mexico, Mexico
[c] School of Health Sciences, Universiti Sains Malaysia, Kelantan, Malaysia
[d] INFORMM, Universiti Sains Malaysia, Kelantan, Malaysia

ARTICLE INFO

Article history:
Received 2 April 2016
Received in revised form
28 July 2016
Accepted 31 July 2016

Keywords:
Mycobacterium smegmatis
Proteoliposomes
Tuberculosis
Vaccines
Challenge

SUMMARY

Tuberculosis (TB) remains an important cause of mortality and morbidity. The TB vaccine, BCG, is not fully protective against the adult form of the disease and is unable to prevent its transmission although it is still useful against severe childhood TB. Hence, the search for new vaccines is of great interest. In a previous study, we have shown that proteoliposomes obtained from *Mycobacterium smegmatis* (PLMs) induced cross reactive humoral and cellular response against *Mycobacterium tuberculosis* (Mtb) antigens. With the objective to evaluate the protective capability of PLMs, a murine model of progressive pulmonary TB was used. Animals immunized with PLMs with and without alum (PLMs/PLMsAL respectively) showed protection compared to non-immunized animals. Mice immunized with PLMsAL induced similar protection as that of BCG. Animals immunized with BCG, PLMs and PLMsAL showed a significant decrease in tissue damage (percentage of pneumonic area/lung) compared to non-immunized animals, with a more prominent effect in BCG vaccinated mice. The protective effect of the administration of PLMs in mice supports its future evaluation as experimental vaccine candidate against Mtb.

1. Introduction

Tuberculosis (TB) remains as one of the most important causes of morbidity and mortality associated with infectious diseases [1–4]. BCG, the vaccine in use for the prevention of TB has a limited impact in the prevention of adult TB and transmission of the disease [5,6]. Thus, multiple strategies are being implemented for the development of new vaccines against TB [7].

The membrane and cell wall components of *Mycobacterium tuberculosis* (Mtb) have been evaluated as experimental vaccine candidates with different strategies, demonstrating their potential for the elicitation of protective immune responses [8–11].

OMV released spontaneously (natural) from mycobacteria, have demonstrated protective capacity in challenge models in mice [10]. In a previous report by our group, OMV obtained from BCG using detergents (Protelolipsomes, PLBCG) showed protection against Mtb in mice (11). The previous results with OMVs, natural or detergent-prepared, demonstrated the potential of these strategies as vaccine candidates against TB [10,11].

Bacterial PL has demonstrated protective capacity against different infectious diseases, as well as adjuvant effects [12–17]. PL from BCG protected mice in a challenge model with Mtb and induced better protection than BCG administered in a prime-boost

Abbreviations: TB, tuberculosis; Mtb, *Mycobacterium tuberculosis*; PL, proteoliposomes; BCG, Bacillus Calmette–Guerin; CFU, Colony Forming Unit; PLMs, proteoliposomes obtained from *Mycobacterium smegmatis*; PLMsAL, PLMs adjuvanted with alum; OMV, outer membrane vesicles.

* Corresponding author.
** Corresponding author. School of Health Sciences, Universiti Sains Malaysia, Kelantan, Malaysia.
E-mail addresses: norazmimn@usm.my (M.-N. Norazmi), ducmar13@gmail.com (A. Acosta).

http://dx.doi.org/10.1016/j.tube.2016.07.017
1472-9792/

scheme [10]. Bioinformatics studies predicted the presence in PLMs of Mtb epitopes expressed during infection in vivo [18]. PLMs are immunogenic and induce cross reactive responses against Mtb in mice [18,19]. Considering these antecedents, the aim of the present work was to evaluate the protective capacity of PLMs in a challenge model with Mtb in mice, demonstrating the protective effect of the administration of this formulation.

2. Material and methods

2.1. Bacterial strains

BCG Moreau strain (Enterprise of Production of Biologicals, Carlos J Finlay, La Habana, Cuba) and *Mycobacterium smegmatis* (Ms) mc^2155 strain [20] were used.

2.2. PLMs

Ms culture was grown in 1% (w/v) yeast extract (Merck, Germany), 0.5% (v/v) glycerol (Riedel de Haen, Germany), 0.05% (v/v) Tween 80 (Fluka, Germany), in 8% (w/v) nutrient broth (Biocen, Cuba) for 48 h with agitation (200 rpm; 37 °C). The purity of the culture was evaluated by Ziehl–Neelsen staining [21]. Proteoliposomes were produced as previously reported [19].

2.3. Mice

Balb/c mice (male, 6–8 weeks) were housed in special microisolator cages coupled to a negative-pressure system.

2.4. Challenge study

The protective capacity of PLMs was studied in an intra-tracheal model of progressive TB in mice. Four groups (n = 4 mice in each group) were inoculated subcutaneously with the following inocula (100 μL): **1. PBS**; **2. BCG**: BCG (single inoculation, 8×10^3 CFU); **3. PLMs:** PLMs (50 μg), and **4. PLMsAL**: PLMs (50 μg) + Alum (1 mg; Alhydrogel, Sigma). Two doses were administered with an interval of 3-weeks in the last two groups. Mice were challenged with 2.5×10^5 CFU of H37Rv Mtb strain in PBS (100 μL) by the intratracheal route, as previously described, two months after the last inoculation [22,23]. Two months later, animals were euthanized under anaesthesia with pentobarbital [22,23] for downstream experiments. Two independent experiments were performed. All procedures were performed in a class III cabinet in a biosafety level III facility following the guidelines of care and use of experimental animals [24] and approved by the Animal Experimentation Ethics Committee of the National Institute of Medical Sciences and Nutrition "Salvador Zubirán" of Mexico.

2.5. Bacilli load

Four lungs per group were homogenized separately in a Polytron homogenizer (Brinkmann Instruments, Rexleid, Canada) in isotonic salt solution (1 ml) containing 0.05% Tween 80 (Sigma). These homogenates (100 μL) were serially diluted (10 fold), plated on Bacto Middlebrook 7H10 agar (Difco, USA) and incubated at 37 °C for Mtb CFU determination. Colonies were counted after 21 days of incubation.

2.6. Histopathology and morphometric studies

The right lung was perfused via the trachea with 100% ethanol (J.T. Baker, Mexico City, Mexico), embedded in paraffin (Oxford Labware, St Louis, MO, USA), sectioned and stained with haematoxylin and eosin (HE). The percentages of the lung surfaces affected by pneumonia were determined using an automated image analyzer (Q Win Leica, Milton Keynes, Cambridge, UK). Briefly, the whole lung was photographed at 25× magnification by a camera system to obtain the image of the total lung which corresponded to the 100% area. Then, the pneumonic patches, which correspond to lung areas with inflammatory infiltrate that occupied alveolar lumens and alveolar-capillary interstitium were delimited and measured with the software analyzer. Finally, the percentage of the lung surface area affected by pneumonia was determined. The percentage data are reported as the mean values ± SD from three different mice at each time-point in two independent experiments.

2.7. Data analyses

One way ANOVA and Multiple Range test were used for the analysis of bacteria burden and pneumonic area in lungs. Measurements were made blind. Data of $\log_{10}$ CFU and percentage of pneumonic area/lung were expressed as the mean ± SD.

3. Results and discussion

PLMs are spherical particles of homogenous diameter, as determined by Transmission electron microscopy and verified by exclusion size chromatography [19] and by spectroscopy of photonic correlation (unpublished results). PLMs contain proteins mainly in the range of 25–67 kDa and the presence of lipids were demonstrated by indirect evidences based on the recognition of Ms lipids by the sera of mice immunized with PLMs [19].

We have previously demonstrated that PLMs elicited cross-reactive responses against Mtb in mice [19]; in this study we evaluated the protective effect of the administration of PLMs in a challenge model with Mtb. The bacterial loads, histopathological changes and the tissue damage in lungs (percentage of pneumonia) were determined in immunized animals compared to mice receiving PBS and BCG.

All the immunized groups showed significant decreases in the bacterial load in lungs compared to mice receiving PBS (Figure 1) ($p < 0.001$). Animals immunized with BCG and PLMsAL had

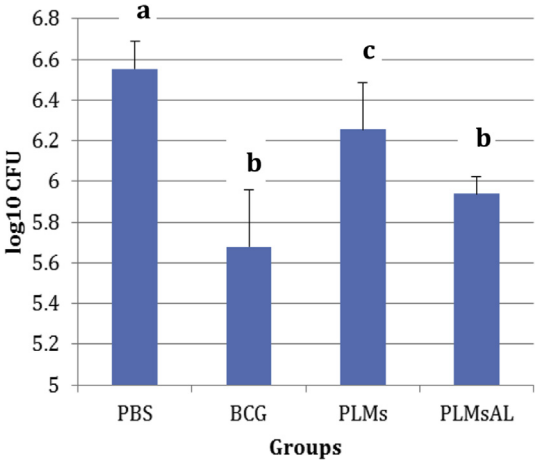

Figure 1. CFU in lungs of mice challenged with Mtb. Groups: **PBS; BCG**: BCG (8×10^3 CFU), one inoculation; **PLMs**: PLMs (50 μg), and **PLMsAL**: PLMs (50 μg) + Alum (1 mg, Alhydrogel, Sigma). Two doses were administered with an interval of 3-weeks in the last two groups. One way ANOVA and Multiple Range test were used for the analysis. Each bar represents the mean ± SD. Different letters denotes significant statistical difference between the groups. $p < 0.001$ (n = 4 per group).