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Vaccination with a low dose of BCG or BCGΔBCG1419c protects against short-term *Mycobacterium tuberculosis* HN878 infection in male CB6F1 mice

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Abstract

Introduction: *Tuberculosis (TB) causes approximately 1.5 million deaths worldwide and 9 million new cases each year. Vaccination with Mycobacterium bovis Bacillus Calmette-Guerin (BCG) is effective in controlling severe forms of TB in childhood, with limited efficacy in preventing lung disease in adults, which is further reduced by infection with Beijing strains. We previously showed that a standard human dose (10^5 colony-forming units [CFU]) of the BCGΔBCG1419c vaccine candidate delayed the progression of lung necrosis in male BALB/c mice infected with Mycobacterium tuberculosis (Mtb) HN878.* **Objective:** *To determine the protective efficacy of a low dose of 2 BCG strains against challenge with a hypervirulent strain of Mtb in a murine model.* **Material and methods***: In this study, we explored the efficacy of vaccination with a low dose (10^2 CFUs) of BCG or BCGΔBCG1419c against Mtb HN878 infection in male CB6F1 mice, both at the short-term (1 month) and chronic (3 months post-infection) stages of active TB.* **Results:** *Vaccination with a low dose of BCG or BCGΔBCG1419c provided protection against Mtb HN878 challenge only 1 month after infection.* **Conclusion:** *Our results have important clinical translational implications, as they raise the hypothesis that the current vaccine may fail to protect humans against Beijing strains if, for example, problems occur during the handling, transportation, or administration of the current BCG vaccine (or a new one, such as BCGΔBCG1419c), leading to a lower than recommended dose being administered.*

Keywords: *Tuberculosis. Mycobacterium tuberculosis. Beijing. HN878. BCG. BCGΔBCG1419c.*

Introduction

Tuberculosis (TB) annually causes over 1 million deaths worldwide, with close to 10 million new cases per year[1]. The current vaccine, Bacillus Calmette-Guerin (BCG), does not provide sterilizing immunity to *Mycobacterium tuberculosis* (Mtb) infection in humans or effectively protects against lung disease. Therefore, a number of potential avenues to improve its efficacy against TB have recently been proposed[2].

Our vaccine candidate called BCGΔBCG1419c, in its second-generation, antibiotic-less version, has been described in its construction and characterization[3]. Vaccination of male BALB/c mice with a human

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standard dose (10^5 colony forming units [CFU]) showed that only BCGΔBCG1419c delayed the progression of TB and reduced lung pathology upon Mtb HN878 intratracheal infection[4].

Based on the findings of Khatri et al.[5], where a low dose of BCG Danish (3×10^2 CFU) reduced the bacillary load of Mtb HN878 in CB6F1 mice, we decided to evaluate whether a low dose of BCG or BCGΔBCG1419c (in the order of 10^2 CFU) would still be effective to protect CB6F1 against TB caused by low dose infection with Mtb HN878.

Material and methods

Culture of mycobacteria and experimental model

We performed culture and infection essentially as we recently described[4] with the difference of dose used here, which was in the 10^2 CFU order for all mycobacteria. Thirty male CB6F1 mice (8-9 weeks old) were purchased from Bioterio Morelos (Mexico) and were randomly allocated into three groups: group 1 received 8.8×10^2 CFU of BCG, group 2 received 5.5×10^2 CFU of BCGΔBCG1419c, and group 3 received phosphate-buffered saline (PBS) (unvaccinated controls). Five mice from each group per time point analyzed were anesthetized with pentobarbital intraperitoneally (210 mg/kg) and were sacrificed by exsanguination at 1- and 3-month post-infection as no animal showed signs of overt disease. The Internal Committee for the Care and Use of Laboratory Animals (CICUAL for its acronym in Spanish) from Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán approved the experiments with project number CICUAL-PAT-2043-21-22-1.

Bacillary load determination and morphometric analysis

These analyses were performed exactly as reported in[4]. For CFU enumeration, the right lung, or whole spleens from mice were mechanically disaggregated with 1 mL of PBS, and 10 mL of the lysate was used for culture on 7H10 oleic acid, dextrose, albumin, and catalase plates. CFU values were Log_{10} transformed before statistical analyses. To evaluate lung damage, left lungs from 3 to 4 mice per group randomly chosen were infiltrated with absolute ethanol, then embedded in paraffin and sliced in sections of 4 mm, then stained with hematoxylin-eosin and Masson's trichrome stain and digitally analyzed in a blind manner by an expert pathologist who analyzed the number of lesions apparent in a section. The percentage of involved parenchyma estimated, as well as peribronchiolitis, perivascular leukocyte infiltration ("perivasculitis"), alveolitis, "granuloma" formation (i.e., granulomatous inflammation), and necrosis on a scale of 0-5 (0 = within normal limits [no change from unaffected tissue]; 1 = minimal changes; 2 = mild changes; 3 = moderate changes; 4 = marked changes; and 5 = very severe changes) were assessed individually. A total lung score was calculated as the arithmetic sum of the scores for each tissue. In addition, a morphometrical analysis of the pneumonic area was performed by calculating the percentage of pneumonia in the sections of 4 mm. The digital analysis was done in Aperio Image Scan Scope and Leica application suite X.

Statistical analyses

Continuous data were analyzed by determining its distribution using a Shapiro-Wilk test and presented as mean plus standard deviation or median plus range when results were normally or not normally distributed, respectively. A Kruskal-Wallis followed by Dunn's test or one-way analysis of variance (ANOVA), and a Tukey *post hoc* test was performed to compare CFU. To compare the scores from the histological analysis, an H-Kruskal-Wallis plus a Dunn *post hoc* test was done. The values of Mtb CFU in lungs were log_{10}-transformed before statistical comparison. Groups where comparisons resulted in $p < 0.05$ were considered statistically different. The analysis was performed in the software GraphPad Prism v9 and Statistical Packages for the Social Sciences for Mac OSX.

Results

Male CB6F1 mice vaccinated with either BCG strain had significantly reduced Mtb HN878 burden in lungs at 1-month post-infection (p.i.) with a mean 0.5-log_{10} drop compared with unvaccinated controls ($p = 0.0026$ and $p = 0.0028$, respectively, one-way ANOVA followed by Tukey's multiple comparisons test, Fig. 1A). This control was lost at 3 months post-infection, as the mean CFU increased by 0.5-log_{10} from month 1 to month 3 p.i. (Fig. 1A). Regarding lung pathology, at 1- and 3-months p.i., only BCGΔBCG1419c reduced the percentage of the pneumonic area although this was not statistically significant ($p = 0.0805$ and $p = 0.1052$, one-way ANOVA followed by Tukey's multiple comparisons test Fig. 1B). For the remaining parameters, we found no statistically significant change for any parameter between vaccinated and unvaccinated mice (Fig. 1C-J).

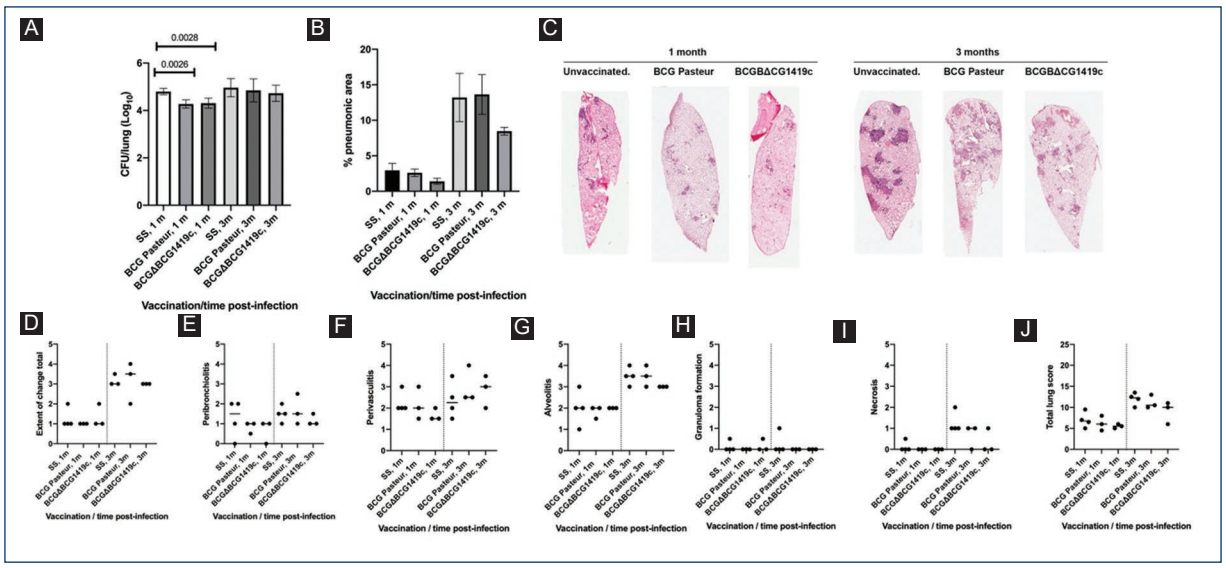

Figure 1. Capacity of BCG or BCGΔBCG1419c to control active (1-month post-infection) or chronic (3 months post-infection) tuberculosis in CB6F1 male mice challenged with a low dose (10^2 Colony-Forming Units) of *Mycobacterium tuberculosis* HN878. **A:** bacillary load in lungs of male CB6F1 vaccinated with either BCG Pasteur or BCGΔBCG1419c. **B:** area of the lung affected by pneumonia, measured by automated morphometry with Leica LAS V4.0 software. **C:** histopathological features of pulmonary TB in a mouse model. Yellow stars: perivasculitis; red star: alveolitis; blue arrow: peribronchiolitis, letter b (bold), bronchiole. BCGΔBCG1419c vaccinated mice showed smaller and well-defined areas of pneumonia with induction of concentric nodular zones in comparison with pneumonia generated in BCG Pasteur vaccine and the unvaccinated control group. **D-J:** detailed histopathological scoring of lesions observed in vaccinated and unvaccinated mice. In all groups, peribronchial and perivenular inflammatory infiltrates and alveolitis were observed. The statistical analysis was carried out with the one-way analysis of variance followed by Tukey's multiple comparisons tests.

Discussion

The results presented in this work suggest that vaccine dose may be relevant for protection against infection with Mtb Beijing strains, as reduction of CFU burden was achieved only at 1-month p.i., as opposed to 3 months p.i. when 10^5 CFU are used to vaccinate BALB/c mice[4]. In support of this notion, other vaccine candidates were shown to be effective in reducing Mtb Beijing strains burden for more than 60 days, but using vaccine doses higher than those used here, such as MTBVAC (10^6 CFU/dose, female C3H/HeNRj mice)[6], and BCGΔureC hly+ (10^6 CFU/dose, BALB/c mice, intravenously administered; mouse sex was not indicated)[7]. Of note, both of these studies used Beijing W strains for challenge, whose virulence as compared with HN878 is presently unknown.

Therefore, we consider that the results presented here raise the hypothesis that a failure to protect against Mtb Beijing strains may arise from the delivery of a lower than recommended dose being administered to vaccinated subjects, which may arise as a consequence of problems occurring during handling, transportation, or administration of the current BCG vaccine (or a new one, such as BCGΔBCG1419c), which alter this vaccine thermal stability; this, in turn, is highly dependent of formulations used to preserve BCG, as it was already demonstrated[8].

In this work, we decided to use CB6F1 mice, a cross between BALB/c and C57BL/6 mice, because these have an increased diversity of major histocompatibility complex class II genes (I-A^b, I-A^d, I-E^d) compared to either of its parental strains, thereby potentially broadening the induction of immunity to infection.

Our results and those reported by Khatri are in agreement, with a few differences: (1) the bacillary load in unvaccinated mice was in the order of 10^5 CFU in this work, compared with 10^6-10^7 in[5], (2) the reduction in mean CFU load was close to 0.5-$\log_{10}$ CFU here, whereas it reached almost 1-$\log_{10}$ in[5]. These differences could be the result of either employing different mouse sex (male here versus female in[5]) or the use of a different BCG strain (Pasteur here versus Danish in[5]). Vaccination dose could also be the source behind the attained burden of Mtb HN878, although this seems unlikely as Khatri et al.[5] did not find a major reduction in CFU when using BCG at doses ranging between 300 and 3×10^5 CFU.

Conclusion

A low dose of BCG or BCGΔBCG1419c was effective in reducing TB disease produced by the highly virulent Mtb HN878 Beijing strain, although only against active and not chronic infection.

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Conflicts of interest

M.A. Flores-Valdez and R. Hernández-Pando are co-inventors on a patent on BCGΔBCG1419c held by the Centro de Investigación y Asistencia en Tecnología y diseño del Estado de Jalisco (CIATEJ), A.C. and the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, which could be construed as a potential conflict of interest. All other authors do not have any conflicts of interest.

Ethical considerations

Protection of humans and animals. The authors declare that the procedures followed complied with the ethical standards of the responsible human experimentation committee and adhered to the World Medical Association and the Declaration of Helsinki. The procedures were approved by the institutional Ethics Committee.

Confidentiality, informed consent, and ethical approval. The study does not involve patient personal data nor requires ethical approval. The SAGER guidelines do not apply.

Declaration on the use of artificial intelligence. The authors declare that no generative artificial intelligence was used in the writing of this manuscript.

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