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BOVINE TUBERCULOSIS

Evaluation of *Mycobacterium bovis* double knockout *mce2-phoP* as candidate vaccine against bovine tuberculosis

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SUMMARY

In this study, a *Mycobacterium bovis* knockout strain in *phoP-phoR* and *mce2* operons was tested as an antituberculosis experimental vaccine in animal models. The double mutant strain was significantly more attenuated than the wild type strain in inmunocompetent and inmunodeficient mice. Vaccination with the double mutant protected mice against challenge with a virulent *M. bovis* strain.

1. Introduction

In the developing countries, the cattle vaccination may represent an intervention strategy to reduce the impact of bovine tuberculosis (BTB) on livestock productivity and human health. To date, however, there is no available vaccine against BTB.

The efficacy of BCG to protect against tuberculosis in humans has shown to be highly variable and dependent on uncertain factors of the tested populations. The field evaluation of the protective efficacy of BCG against bovine tuberculosis has indicated that BCG is unable to provide 100% protection [4,13]. An attractive strategy to overcome the incomplete protection afforded by BCG is to delete specific genes from *Mycobacterium bovis* (the causative agent of BTB). The target genes would be those that are not involved in protective immunity but are relevant for its virulence. However, little is known about the precise mechanism of host-pathogen interactions that result in protection against BTB and about the genes/factors involved in this protective immune response. As a result, the identification of those ideal gene targets whose elimination from mycobacterial genome attenuates the virulence without compromising the immunogenic properties of the candidate vaccine strains has not been successful so far.

Therefore, the common strategy undertaken in most TB vaccine development has been to delete virulence genes and then experimentally test the protection afforded by the attenuated mutant strains in animal models. Following this rationale, many mutant strains have been evaluated as experimental tuberculosis (TB) vaccines but few were developed and tested as candidate vaccine against BTB. In a previous study we reported that a *M. bovis* knock out in *mce2A* and *mce2* genes from the *mce2* operon (MbΔmce2) conferred better protection than BCG in a cattle model of BTB [2]. Although we have previously observed that MbΔmce2 was unable to produce tuberculosis in cattle [3], which suggests that in cattle this is a safety vaccine, the Geneva consensus criteria [7,12] have established that TB vaccine must be based on two independent stable deletion mutations without antibiotic resistance markers. Therefore, to fulfil part of the requirements for a live TB candidate vaccine, we deleted a second virulence gene from MbΔmce2 [2]. Arbues and collaborators obtained successful results using a *Mycobacterium tuberculosis* mutant in the two-component system PhoP-PhoR as a TB vaccine candidate [1]. For this reason we chose this two-component system as knockout target in the MbΔmce2 strain.

2. Results

2.1. Knockout of phoP in MbΔmce2 strain

The *phoP* gene (Rv0757) in MbΔmce2 [3] genome was knocked out by replacing bases 119 to 680 of the gene with a hygromycin-resistance cassette. The deletion of *phoP* from MbΔmce2 was then confirmed by polymerase chain reaction (PCR) and Southern blot (Supplementary material S1). The growth profiles of the wild type and mutant strain under in vitro standard culture conditions showed similar doubling times (data not shown). Thus, the mutation of *phoP* does not appear to compromise the in vitro growth of

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M. bovis. Following the same experimental strategy, we deleted the *phop* gene from the wild type *M. bovis* as well. This last mutant strain was used as a control in the protection experiment (see below) of this study.

2.2. Examination of MbΔmce2-phoP virulence in BALB/c and nude mice

First, we assessed the in vivo virulence of MbΔmce2-phoP in immunocompetent mice. To this purpose, we used the intratracheal route to infect BALB/c mice and determined lung colonization by counting the bacterial colony forming units (CFU). BALB/c mice (15 per group) were infected with 1×10^5 CFU of the MbΔmce2-phoP or the wild type strains. At 60 days post-infection, the number of CFU in the lungs of animals inoculated with MbΔmce2-phoP was significantly lower than those of the wild type strain ($p < 0.001$) (Figure 1A).

Then, we compared the virulence of MbΔmce2-phoP mutant to that of the BCG and the wild type in immunodeficient mice in order to comply with the safety requirements for a live TB candidate vaccine. Nude mice (10 per group) were intratracheally infected with 5×10^4 CFU of MbΔmce2-phoP, the wild type *M. bovis* or BCG strains, and the survival was subsequently assessed. The median survival of MbΔmce2-phoP (150 days) was statistically different ($p < 0.001$) to that of the BCG-infected animals (252 days) (Figure 1B). When compared to the wild type strain, the double mutant MbΔmce2-phoP was significantly more attenuated.

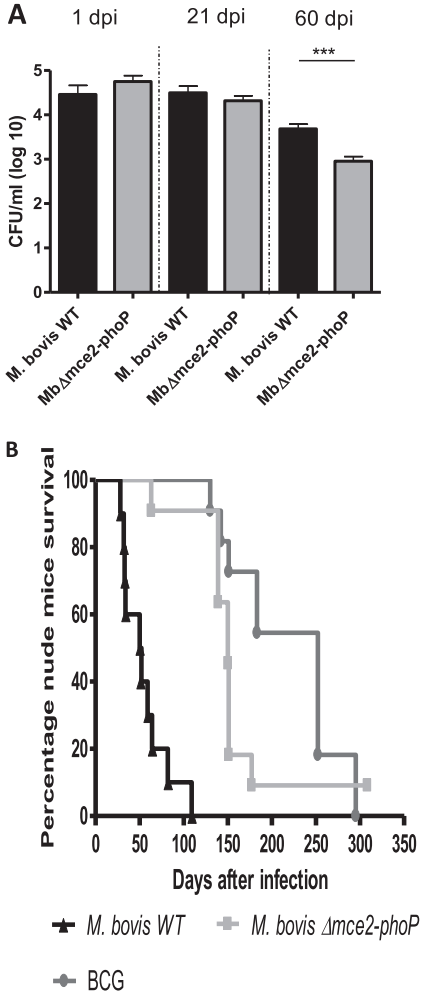

Figure 1. Virulence assays in mice. A) Replication of *M. bovis* strains in BALB/c mouse organs after intratracheal inoculation (1×10^5 CFU). The results are expressed as the mean of five mice. Data were analysed using one-way ANOVA analysis and Bonferroni's post-test. (***$p < 0.001$). B) Survival of nude mice after intratracheal inoculation *M. bovis* strains (5×10^4 CFU). Statistical analysis was performed using Mantel-Cox test. dpi: days post infection, CFU: colony forming units.

2.3. Evaluation of MbΔmce2-phoP as a BTB vaccine candidate in a mouse model

All immunized mice were protected compared to the saline control ($p < 0.001$). The protection conferred by the MbΔmce2-phoP mutant was equivalent to that of BCG in lungs and spleens (Figure 2A). At the end of the experiment, the CFU of the vaccine strains (MbΔmce2-phoP and BCG) in mouse spleens was very low and equivalent between groups (data not shown). This result indicates that the virulence of MbΔmce2-phoP is similar to that of BCG in immunocompetent mice. However, comparing to our previous unpublished results, the bacterial load of the double mutant strain in lungs of immunocompetent BALB/c mice was significantly higher than that of mice infected with BCG Pasteur (at 60 dpi: 900 CFU ± 113 vs 244 CFU ± 40.06, respectively). To assess if either the *mce2* or *phoP/phoR* loci encode factors that are relevant to induce immune protection, we also evaluated each single mutant at each locus as candidate vaccines. Figure 2B shows that all mutant strains conferred similar protection against *M. bovis* challenge in this mouse model.

The analysis of the immune response induced by the mutant strains at 30 days post vaccination showed that all mouse groups elicited a Th1 cytokine profile after the stimulation with *M. bovis* antigens (PPDb), with no statistical differences among the candidate mutant strains and also when compared to the BCG vaccinated group (Supplementary material S2).

3. Discussion

The *phoP-phoR* operon of *M. tuberculosis* encodes PhoR, a histidine protein kinase (sensor of stimuli), and PhoP, a transcriptional regulator that receives a phosphate from PhoR. First thought to be implicated in phosphate metabolism and transport, the two-component system PhoP-PhoR controls diverse aspects of the metabolic physiology of *M. tuberculosis*. As described by [5] in their review, many genes involved in lipid metabolism are regulated by PhoP-PhoR, such as *pks3* (polyketide β-ketoacyl synthase), *pks5* (polyketide synthase), *papA3* (polyketide synthase associated protein), *fadD26* (a fatty acid-coenzyme A ligase), *lipF* (an esterase/lipase), *fbpA* (secreting type Ag85A FbpA), *mmpL10* and *mmpL8* (transport proteins belonging to the RND superfamily). Therefore, *M. tuberculosis phoP* mutants produce less quantity of sulfolipids, diacyltrehaloses, and polyacyltrehaloses. Furthermore, an attenuated *M. tuberculosis*, the H37Ra strain, also produces less quantities of these types of lipids due to a single nucleotide mutation within a DNA binding domain of PhoP that abolishes the binding of PhoP to its own promoter. PhoP also regulates genes related to ESX-1 secretion system required for *M. tuberculosis* complex virulence. As a result, the secretion of both ESAT6 and CFP10 is significantly reduced in *M. tuberculosis* H37Rv ΔphoP and *M. tuberculosis* H37Ra. These findings evidence the critical role of PhoP-PhoR in the metabolic control of *M. tuberculosis* virulence factors. In line with these findings, the *phoP* and *phoP-phoR* mutants of *M. tuberculosis* are attenuated for growth in various cultured or primary cell types, such as murine bone marrow-derived macrophages, murine alveolar macrophages, murine J774 macrophage cells, human THP-1 cells, as well as in BALB/c mice and guinea pigs [5].

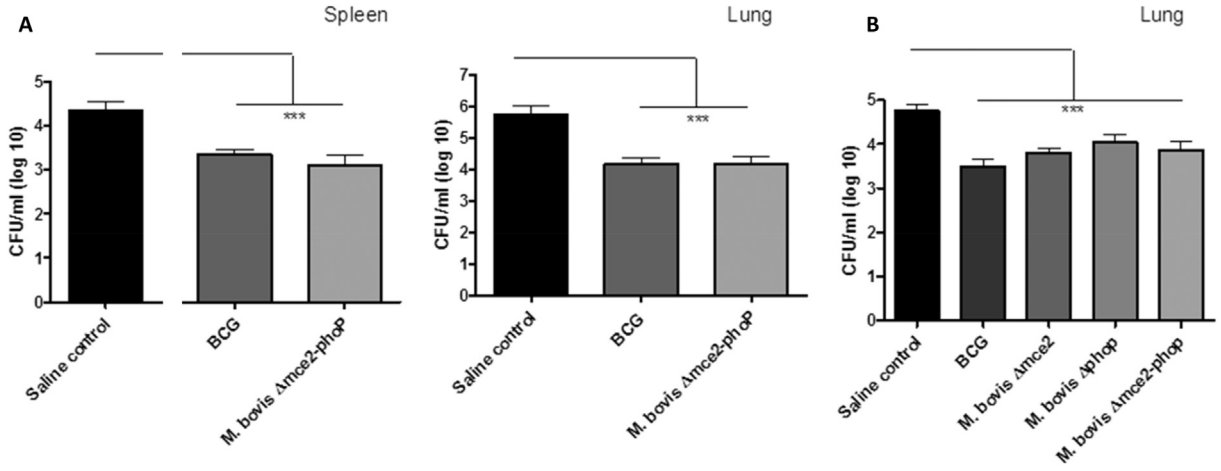

Figure 2. Protection assays in BALB/c mice. Groups of seven mice were vaccinated with 1×10^5 CFU of BCG or mutant strains, and compared with control nonvaccinated animals. 30 (A) or 55 (B) days post-vaccination, mice were challenged by intratracheal route with 5×10^4 CFU of *M. bovis* and one month later the bacterial burden was determined in spleens and lungs. Data were analysed using one-way ANOVA analysis and Bonferroni's post-test. (***$p < 0.001$).

In this study we demonstrated that the double mutant strain significantly reduces its virulence in both immunocompetent and immunodeficient mice. However, opposite to the findings observed for the vaccine candidate *M. tuberculosis phoP* mutant SO2 [8], the double *M. bovis* mutant killed immunodeficient mice faster than BCG. This stronger virulence may obey to the following reasons: i. differences in the immunodeficient mouse models, since Δmce2-phoP was assayed in Nude mice, while *M. tuberculosis* ΔphoP was assessed in SCID mice; ii. the magnitude in controlling the expression of virulence genes of PhoP-PhoR may be lower in *M. bovis* than in *M. tuberculosis*. In fact, it has been recently reported that three mutations affect the two-component virulence regulation system PhoPR in *M. bovis* and in the closely linked *Mycobacterium africanum* lineage 6. The authors have found that the specific mutations present in the phoPR-bovis allele negatively impact in the expression of certain PhoPR-regulated genes and in the virulence of the recombinant strain when transferred into *M. tuberculosis* [6,10].

The vaccination of mice with the single and double mutants provided equal protection to BCG. This result is consistent with that of [8]; in which the BALB/c mice vaccinated with the *M. tuberculosis phoP* mutant strain SO2 showed similar protection against *M. tuberculosis* challenge to that vaccinated with BCG. Moreover, in the present study, the vaccine candidate MbΔmce2 was also equally effective than BCG in protecting mice against *M. bovis* challenge. Altogether, these results indicate that BALB/c mice are only suitable for a first testing/screening of TB or BTB vaccine candidates. Further studies in other animal models, such as guinea pigs and cattle, are required to assess the benefit of using these vaccine candidates, instead of BCG, for protection against BTB.

In conclusion, the results of the protection conferred by MbΔmce2-phoP strain in mice postulate this double mutant strain as a potential tuberculosis vaccine candidate to be used in cattle.

4. Material and methods

4.1. Construction and characterization of Mycobacterium bovis mutants

Genomic regions of about 2 kb either upstream or downstream of *phoP* were obtained by PCR from *M. bovis* total DNA by using the following pairs of primers: Pair 1: 5′actagtaacccaaaacgtcaagcatc and 5′actagtggctcaccgacagcagttc; Pair 2: 5′tctagatggggagaagcggctgctg and 5′tctagaggtgtgctgtatggcatttg. The amplified fragments were cloned separately into pPR27 shuttle vector [9], in which a hygromycin resistant cassette had been previously inserted into Xba1 and Spe1 sites. This plasmid was used to electroporate either *M. bovis* NCTC 10772 or the mutant MbΔmce2 [3]. The selection of the mutant strains was performed as previously described [9].

Chromosomal DNA was prepared from the selected colonies according to van Soolingen et al. [11], digested with either EcoRV or NheI and then analysed by Southern blotting, by using the wild type gene as probe. The allelic replacement in the candidate mutant clones was then confirmed by PCR using the primers: 5′cgtgactcgctacaggacaa and 5′ gacgacgttgacatcaccac.

4.2. Mouse vaccination and infections

Groups of female nude (N:NIH (S)-*Foxnl*^{nu}) or BALB/c mice (repeated twice) of 6–8 weeks of age were used to assess the virulence of the *M. bovis* strains. The survival curve of the nude mice infected with the wild type strain was performed separately.

Female BALB/c mice ranging in age from 6 to 8 weeks were used for vaccination and challenge experiments. BCG Pasteur was used as the control vaccine. This experiment was repeated twice. A mouse group vaccinated with a BCG expressing the green fluorescent protein was also included. This vaccine conferred the same protection than untransformed BCG Pasteur (data not shown). The vaccine candidates were delivered subcutaneously in a single dose. After vaccination, the animals were infected with *M. bovis* NCTC 10772 by intratracheal instillation. Finally, 30 days after challenge all mice were sacrificed and CFU were determined in organs.

4.3. Immune responses

BALB/c mice (seven per group) were vaccinated via subcutaneous route with 1×10^5 CFU of each strain. After 45 days of the immunization, splenocytes were cultured (2×10^6 cells/well) either with or without purified protein derivatives bovine (PPDB). After 72 h of incubation, different cytokines (IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17A and IL-10) were quantified in culture supernatants using a Th1/Th2/Th17 cytokine kit (BC, cat. 560485) and a flow cytometer.

Experiments with mice were performed in compliance with the regulations of the Institutional Animal Care and Use Committee (CICUAE) of INTA.

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Competing interests: None declared.

Ethical approval: Not required.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2015.01.001

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