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BOVINE TUBERCULOSIS

Mycobacterium bovis Δmce2 double deletion mutant protects cattle against challenge with virulent *M. bovis*

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SUMMARY

A *Mycobacterium bovis* strain deleted in *mce2A* and *mce2B* genes (*M. bovis* Δmce2) was tested as an experimental vaccine in cattle challenged with a virulent *M. bovis* strain. Three-and-a-half-month old calves ($n = 5$ to 6 per group) were vaccinated and challenged with a virulent strain of *M. bovis* by the intratracheal route 9 weeks after vaccination. A non-vaccinated group and a group vaccinated with BCG were included as controls. Blood samples were collected to measure IFN-γ by an interferon-gamma release assay (IGRA), cytometry and cytokine responses of bovine purified protein derivative (PPD) restimulated peripheral blood mononuclear cells (PBMCs). The IGRA test showed IFN-γ values similar to pre-vaccination except for the animals vaccinated with *M. bovis* Δmce2, where a significant increase was observed at 30 days post-vaccination. The expression of IL-2R on $CD4^+$ cells in response to PPD from the animals vaccinated with Δmce2 increased at 15 days post-vaccination compared to cells from non-vaccinated group. Vaccination of cattle with *M. bovis* Δmce2 induced the highest ($P < 0.05$) expression of IFN-γ and IL-17 mRNA upon PPD stimulation of PBMCs compared to vaccination with BCG or that for the non-vaccinated group. There was a weak positive correlation between the production of these proinflammatory cytokines post-vaccination and reduced pathology scores post-challenge. The animals were euthanized and necropsied 100 days after challenge. The group vaccinated with *M. bovis* Δmce2 displayed a significantly lower histopathological score for lesions in lungs and pulmonary lymph nodes than for the other groups ($P < 0.05$). A marked positive reaction to tuberculin intradermal test was observed post-vaccination in animals vaccinated with *M. bovis* Δmce2 compared to those vaccinated with BCG or the non-vaccinated group. In contrast, after challenge, non-vaccinated animals had greater skin test responses than the vaccinated animals. In summary, *M. bovis* Δmce2 is a promising vaccine candidate to control *M. bovis* pathogenesis in cattle.

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1. Introduction

Bovine tuberculosis (BTB) is an infectious disease caused by *Mycobacterium bovis*, which affects cattle as well as a wide range of other mammals, including humans.[1] *M. bovis* is a member of the *Mycobacterium tuberculosis* complex (MTBC), which also includes *M. tuberculosis, Mycobacterium africanum, Mycobacterium canetti, Mycobacterium microti, Mycobacterium caprae* and *Mycobacterium pinnipedii.*

In Latin America, especially in South America, meat and milk are essential products for internal consumption and exportation. Therefore, eliminating diseases that limit the trade of animals and their products is a priority in this region. The population of cattle in

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Latin America has been estimated in 450 million head, 51 in Argentina, 189 in Brazil, 4.1 in Chile, 13.5 in Venezuela and 30 in Mexico.[2] About 262 million cattle live in countries with more than 1% prevalence of BTB.[2]

Conventionally, the control of BTB is based on a test and slaughter strategy, which has reduced the incidence and prevalence of the disease in developed countries, except in those with wildlife reservoirs such as the United Kingdom and New Zealand. However, this method is costly and lengthy to be applicable to most of the developing world. Vaccination of cattle represents an alternative intervention strategy to reduce the impact of BTB on livestock productivity and human health in developing countries. The only currently available vaccine against human tuberculosis is *M. bovis* bacillus Calmette-Guérin (BCG). BCG and other vaccines have been used in cattle in a large number of experiments and trials with variable efficacies as reviewed by Buddle et al.[3] and Waters et al.[4]

The construction of *M. tuberculosis* mutants has allowed the identification of several genes responsible for *M. tuberculosis* complex pathogenicity. These mutants are then tested for multiplication in the lungs of mice or guinea pigs. Several studies using this functional genomic approach have reported the development of gene knock-out mutants with different levels of attenuation, leading to the concept that rationally-attenuated live and replicating mutants of *M. tuberculosis* are potential vaccine candidates against tuberculosis.[5,6] Whereas the vaccine currently in use, BCG, lacks more than 100 genes compared to its parent strain, the use of *M. tuberculosis* complex strains attenuated in virulence genes provides the advantage that only a few selected genes are targeted, making them better candidates as vaccines.[7,8]

In 1993, the description of a DNA fragment from *M. tuberculosis* that encodes proteins mediating entry into mammalian cells led to the discovery of the *mce* operon[9] which is now recognized as a group of four major operons. While the virulence of *mce* mutants in mouse infection models seems to differ depending both on the route and dose, along with the susceptibility of the mouse strain used, in the majority of studies loss of one or more of the *mce* operons usually results in attenuation.[10–14] In one case, however, an attenuated mutant was still able to cause reactivation.[15]

In this study we investigated the capacity of a *M. bovis* strain deleted in two genes in the *mce2* operon, *mce2A* and *mce2B*, to protect cattle against an intratracheal challenge of a virulent *M. bovis* strain, as previously determined in mice and cattle virulence assays.[16,17] The mutant strain protected these animals, as shown by reduced lung pathology, compared to unprotected controls. This candidate is thus a new potential vaccine that should be further tested.

2. Methods

2.1. Bacterial strains and culture media

Δmce2 was derived from *M. bovis* NCTC 10772 as previously described.[18] The strain used for challenge was *M. bovis* 04-303, which is an isolate obtained from a wild boar with tuberculous lesions and has been shown to produce tuberculous lesions in cattle.[17] Both isolates were grown at 37 °C in Middlebrook 7H9 (BD, USA) liquid medium plus 0.5% Tween 80 enriched with 0.4% pyruvic acid, and 1% albumin dextrose complex (ADC). Viable bacteria in the inocula were enumerated with the Live/Dead BacLight™ Bacterial Viability kit (Invitrogen, Molecular Probes, Carlsbad, California).

2.2. Vaccination, challenge and sampling schedule

All the animals used in this study were negative to the tuberculin skin test and showed absence of *in vitro* gamma interferon (IFN-γ) response to both avian tuberculin PPD (PPDA) and bovine tuberculin PPD (PPDB) at the beginning of the experiment. The results shown in this study are part of a trial that included another candidate vaccine whose results will be separately published. Animal experimentations were performed inside the biosafety BSL3 facilities for large animals of the National Institute of Agricultural Technology (INTA), Argentina, in compliance with the regulations of the Ethical Committee of INTA (CICUAE) and the biosafety protocols as authorized by the National Service of Agricultural and Food Health and Quality (SENASA). Three groups of five to six Holstein-Fresian calves (3–4 months of age) were inoculated subcutaneously in the side of the neck with 10^6 colony-forming units (CFU) of either *M. bovis* Δmce2 or BCG Pasteur 1173P2 suspended in phosphate buffered saline (PBS). Sixty days after vaccination, animals were infected with an *M. bovis* 04-303 field strain by intratracheal instillation of 10^6 CFU as described previously.[17] The previous study established that this was an appropriate dose to produce reproducible tuberculous lesions in cattle.[17] The schedule is shown in Figure 1.

2.3. IGRA interferon gamma (IFN-γ) release assay

Heparinized blood samples were dispensed in 200 μl aliquots into individual wells of a 96-well plate. Wells contained whole blood plus 20 μg/ml *M. bovis* PPD (Prionics, Switzerland), 20 μg/ml *Mycobacterium avium* PPD (Prionics), or 4 μg/ml of ESAT6 or CFP10 recombinant antigens. Blood cultures were incubated for 18 h and plasma was harvested and stored at −80 °C. IFN-γ concentrations in stimulated plasma were determined using a commercial ELISA-based kit (Bovigam™; Prionics). Absorbance of standards and test samples were read at 450 nm. The optical density (OD) for the PBS controls, which was usually approximately 0.1 OD units, was used to normalize individual readouts and to calculate optical density indexes (ODIs), where the results obtained by antigen stimulation were divided by the results for the PBS-stimulated cultures. Duplicate samples for individual antigens were analyzed.

2.4. Necropsy

The animals were euthanized with the permission of the Institutional Animal Care and Use Committee (IACUC-CICVyA) of the Centro Nacional de Investigaciones Agropecuarias, Instituto Nacional de Tecnología Agropecuaria 100 days post-challenge. Necropsy was conducted paying special attention to typical tuberculosis lesions at lungs, liver, pulmonary lymph nodes (tracheobronchial and mediastinal lymph nodes), prescapular lymph nodes and cranial lymph nodes (submandibular, retropharyngeal, and parotid) were examined and sampled. Lymph nodes were systematically examined and kept for subsequent analysis irrespective of the presence or absence of macroscopic lesions in these organs. Macroscopic lesions were converted to scores according to Wedlock et al.[19] Two pieces were collected from every sample, one for microbiological culture and PCR and the other for histological analysis.

2.5. Cytometry

For flow cytometry determinations, 2×10^6 cells were incubated either with or without PPDB. To evaluate the expression of CD4 (MCA 1653A647, IgG2a), CD8 (MCA837PE, IgG2a), and CD25 (MCA2430F and MCA2430PE) surface markers, cells were stained with fluorescent-conjugated monoclonal antibodies (AdDSerotec, Oxford, UK). Stained cells were analyzed in a FACScalibur cytometer (BD, Franklin Lakes, NJ, USA) using Cell Quest software. Analysis gates were set on lymphocytes according to forward and side scatter. The expression of IL-2R (CD25) as a marker of T cells activation was analyzed in $CD4^+$ and $CD8^+$ populations. Percentages of

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