Research Article

Assessment of Mycobacterium bovis Deleted in p27-p55 Virulence Operon as Candidate Vaccine against Tuberculosis in Animal Models

Maria V. Bianco,1 Simon Clark,2 Federico C. Blanco,1 Sergio Garbaccio,3 Elizabeth García,1 Angel A. Cataldi,1 Ann Williams,2 and Fabiana Bigi1

1 Instituto de Biotecnología, CICyA-INTA, N. Repetto and De los Reseros, 1686 Hurlingham, Argentina
2 Public Health England, Porton Down, Salisbury SP4 0JG, UK
3 Instituto de Patobiología, CICyA-INTA, N. Repetto and De los Reseros, 1686 Hurlingham, Argentina

Correspondence should be addressed to Fabiana Bigi; fbigi@cnia.inta.gov.ar

Received 10 October 2013; Revised 21 November 2013; Accepted 28 November 2013; Published 21 January 2014

A Mycobacterium bovis knockout in p27-p55 operon was tested as an antituberculosis experimental vaccine in animal models. The mutant MbΔp27-p55 was significantly more attenuated in nude mice than its parental strain but more virulent than BCG Pasteur. Challenge experiments in mice and guinea pigs using M. bovis or M. tuberculosis strains showed similar protection conferred by MbΔp27-p55 mutant than BCG in terms of pathology and bacterial loads in spleen but lower protection than BCG in lungs. When tested in cattle, MbΔp27-p55 did not induce IL-2 expression and induced a very low production of IFNγ, suggesting that the lack of P27/P55 reduces the capacity of M. bovis of triggering an adequate Th1 response.

1. Introduction

Mycobacterium bovis (Mb), the causative agent of bovine tuberculosis (BTB), infects cattle and other animals, including humans [1]. Although vaccination of cattle may represent an intervention strategy to reduce the impact of BTB on livestock productivity and human health in the developing countries, to date there is no available vaccine against BTB.

The gene that encodes P27/LprG constitutes a virulence operon together with p55 that encodes an efflux pump or transporter [2]. Although P27 induces Th1 immune response, in BALB/c mice, when administrated as vaccine with BCG produced an adverse effect [2] Moreover, coadministration of P27 with M. tuberculosis aggravates the infection [2], suggesting that this protein plays a role in M. tuberculosis infection by inducing increased suppression of the immune response. In this study we investigated the capacity of a M. bovis strain knockout in p27-p55 operon to induce protective immune response in cattle and to vaccinate mice and guinea pigs against infection with virulent M. bovis and M. tuberculosis, respectively.

2. Results and Discussion

2.1. Examination of MbΔp27-p55 Virulence in Nude Mice. In order to comply with the safety requirements for a live TB candidate vaccine we evaluated the virulence of the MbΔp27-p55 in immunodeficient mice. Nude mice (10 per group) were infected with 12,500 colony forming units (CFUs) of the wild type or MbΔp27-p55 strains, and survival was assessed. The median survival of wild type-infected mice (59 days) was statistically different (P < 0.001) to that of MbΔp27-p55-infected animals (109 days) (Figure 1). This result demonstrates that MbΔp27-p55 is attenuated in the absence of a T-cell adaptive immune response and therefore is a safe candidate to be tested as a TB vaccine.

2.2. Evaluation of MbΔp27-p55 as TB Vaccine Candidate in Animal Models. The experimental challenge model of progressive pulmonary tuberculosis was used in this study [3]. Groups of BALB/c mice (7 per group) were vaccinated subcutaneously in the base of the tail with 100,000 bacilli of either the MbΔp27-p55 mutant or BCG Pasteur. At 60 days
after-vaccination, all mice were challenged intratracheally with 125,000 CFUs of a virulent *M. bovis* strain. Mice were then killed at 30 days after-challenge. Levels of protection were determined by evaluating the numbers of viable *M. bovis* strain bacilli recovered from lungs and spleen. The numbers of CFUs cultured from the organs of each group are shown in Figure 2(a). Mice vaccinated with either BCG or MbΔp27-p55 were protected compared to saline control (*P < 0.001*), in both lungs and spleen. However, in the lungs, the protection conferred by the mutant was statistically lower than that of BCG (*P < 0.05*).

Groups of 8 Dunkin-Hartley guinea pigs were used to evaluate the efficacy of MbΔp27-p55 compared with BCG Danish 1331 both delivered subcutaneously in a single dose at a concentration of 5 × 10⁴ CFU. Twelve days after immunization, animals were infected with a low aerosol dose of *M. tuberculosis* H37Rv. At 4 weeks after-challenge, guinea pigs were killed and organs were removed.

Protection was primarily assessed by measuring bacterial load in lungs and spleen and comparing the vaccinated groups of animals with the control group (saline). Guinea pigs vaccinated with either BCG or MbΔp27-p55 were protected compared to saline control (*P < 0.001*), in both lungs and spleen. However, in the lungs, the protection conferred by the mutant was statistically lower than that of BCG (Figure 2(b)).

A histopathological analysis of lungs and spleen lesions revealed that both vaccinated groups (MbΔp27-p55 and BCG) showed significantly reduced consolidation, foci of necrosis/caseation, and foci of calcification when compared with the unvaccinated group (Figure 2(c)). Again, guinea pigs vaccinated with BCG showed significantly reduced lung pathology when compared to animals vaccinated with the mutant strain.

### 2.3. Assessment of the Immune Responses Induced in Cattle after Inoculation of a *M. bovis* Strain Deleted in p27 and p55 Genes

In order to better understand the failure of MbΔp27-p55 to protect both mice and guinea pigs against tuberculosis, we used the cattle model to evaluate the immune response induced after infection with this mutant strain.

In peripheral blood monocyte cells (PBMCs) isolated 90 days after infection with the wild type strain, activation of CD4+ cells increased upon stimulation with PPDB (*P < 0.01*) (Figure 3(a)). In contrast, PBMCs isolated from animals infected with MbΔp27-p55 did not respond to specific stimulation with activation of CD4+ cells in any time point assayed.

We assessed the cytokine expression profile in PBMCs by measuring cytokine mRNAs after stimulating the cells with PPDB (Figure 3(b)). Values for sequential samples were normalized to values before inoculation for each animal. Given that there are available ELISA commercial assays to detect bovine IFNγ we used this methodology instead of quantification of IFNγ mRNA by RT-qPCR.

At 90 days after-infection (dpi), the expression of interleukin-2 (IL-2) in PBMCs was upregulated only in the group infected with the wild type strain, which is consistent with the CD4+ proliferative response, detected only in this animal group. Unexpectedly, only the group inoculated with the mutant responded to PPDB stimulation with production of IL-12 (*P < 0.05*), while the expression of TNFα was upregulated in both animal groups with no significant differences between them (Figure 3(b)).

The expression of IL-4, a Th2 cytokine, was downregulated in both groups (Figure 3(b)). This result is consistent with the low level of IFNγ detected at 90 dpi in both animal groups. It has been proposed that IL-4 is produced to compensate the inflammatory response induced by IFNγ.

At 120 dpi, the production of IFNγ in culture supernatant of PBMC stimulated with PPDB was significantly lower in the group inoculated with the MbΔp27-p55 mutant than in the group inoculated with the wild type strain. In fact, the group inoculated with the mutant strain produced very low quantities of IFNγ after PPDB stimulation throughout this study (Figure 3(c)).

Altogether, these results indicate that, although there is upregulation of IL-12 and TNFα, observed at 90 dpi, the lack of P27 and P25 in *M. bovis* reduces the capacity of the bacilli to induce a significant Th1 response when inoculated in cattle.

### 3. Conclusions

In this study we demonstrated that a *M. bovis* mutant in p27-p55 operon did not confer better protection than BCG in both mice and guinea pigs. MbΔp27-p55 was more virulent than BCG in athymic mice, suggesting that its reduced protective capacity was not due to an inability to establish an infection. We found that the mutant induced in cattle the transcription of IL-12 and TNFα, two important Th1 cytokines. However, in contraposition, CD4+ cells from cattle inoculated with the
mutant did not proliferate in response to specific stimuli, and the production of IFNγ in blood was nearly undetectable in this animal group. Therefore, altogether these results suggest that the lack of p27-p55 operon reduces the capacity of M. bovis to induce an adequate Th1 response, underlining the immunogenic properties of P27. In the light of the results of this study, a M. bovis deleted in p55 virulence gene carrying an intact p27 gene would be an attractive candidate to be tested as TB vaccine.

4. Materials and Methods

4.1 Mouse Vaccination and Infections. Groups of female nude (N:NIH (S)-Foxn1null) mice of 6–8 weeks old were used to assess the virulence of MbΔp27-p55 strain.

BALB/c mice aging 6–8 weeks old were used for vaccination and challenge experiments. M. bovis NCTC 10772 strain (the parental strain of mutant MbΔp27-p55) was used as challenge strain. This experiment was repeated twice.
Figure 3: Continued.
Experiments with mice were performed in compliance with the regulations of Institutional Animal Care and Use Committee (CICUAE) of INTA. MbΔp27-p55 or BCG Pasteur were delivered subcutaneously in a single dose at a concentration of 1 × 10⁶ CFU. 60 days after vaccination the animals were infected with 1,25 × 10⁵ CFU of M. bovis by intratracheally instillation.

4.2. Guinea Pig Vaccination and Infection. Groups of 8 Dunkin-Hartley guinea pigs, weighing between 250 and 300 g (free of infection), obtained from a commercial supplier (Harlan, UK), were used to evaluate the efficacy of MbΔp27-p55 compared with BCG Danish 1331 (Statens Serum Institute, Copenhagen, Denmark), both delivered subcutaneously in a single dose at a concentration of 5 × 10⁵ CFU, and a negative control unvaccinated group. Guinea pig experimental work was conducted according to UK Home Office legislation for animal experimentation and was approved by the local ethics committee.

Animals were infected with a low aerosol dose (10–50 CFU retained dose in the lung) of M. tuberculosis H37Rv [4] 12 weeks after vaccination. Nose only aerosol challenge was performed using a fully contained Henderson apparatus as previously described [5, 6] in conjunction with the AeroMP (Biaera) control unit [7]. The aerosol was generated from a water suspension containing 5 × 10⁸ CFU/ml in order to obtain an estimated retained, inhaled dose of approximately 10–50 CFU/lung [7]. At 4 weeks after challenge, guinea pigs were killed humanely by intraperitoneal injection of pentobarbital (Euthatal). Postmortem, lungs and spleens were taken and processed for bacteriology and histopathology analysis (subjective score), as described previously [8]. A significant reduction in CFU (bacterial load) and the nature and severity of the lesions (histopathology score) of vaccinated animals when compared with the control groups was considered a protective effect of the vaccine [9].

4.3. Cattle Infections and Immune Response Evaluations. Cattle infections were performed in compliance with the regulations of CICUAE and authorized by the National Service of Agricultural and Food Health and Quality (SENASA) and National Consultant Commission of Agricultural Biotechnology (CONABIA). Group of Holstein-Fresian calves (six months old) were inoculated intratracheally as described previously [10] with 10⁴–10⁵ CFUs of either M. bovis NCTC 10772 (N = 4-5) or MbΔp27-p55 (N = 4) and blood samples were taken at different points. After three months of infection, the calves were euthanized and then thin slices of lungs and lymph nodes of the head and pulmonary region were analyzed for granuloma formations. Only one of the animals inoculated with the wild type NCTC 10772 strain developed macroscopic lesions compatible with tuberculosis (data not shown). These lesions were located in retropharyngeal lymph nodes. No lesions were observed in animals inoculated with the mutant MbΔp27-p55. Flow cytometry determinations and cytokine expression analysis were performed as previously described [10]. IGRA Interferon Gamma (IFN-γ) release assays were performed on blood samples by using ELISA-based kit (Bovigam; Prionics) as previously described [10]. Duplicate samples for individual antigens were analyzed.
Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors gratefully acknowledge the support from the Biological Investigations Group at PHE, Porton Down, United Kingdom. The views expressed in this paper are those of the authors and not necessarily those of the NHS, the National Institute for Health Research, or the Department of Health. The present study was supported by Fonarsec Agro 02/10 and INTA Grant PNBI0I131034. Fabiana Bigi and Angel A. Cataldi are CONICET Fellows. The authors thank Valeria Rocha, Javier Leiva, and Diego Soreira for technical assistance.

References

[1] F. Biet, M. L. Boschirol, M. F. Thorel, and L. A. Guilloteau, “Zoonotic aspects of Mycobacterium bovis and Mycobacterium avium-intracellulare complex (MAC),” Veterinary Research, vol. 36, no. 3, pp. 411–436, 2005.
[2] M. A. Forrellad, L. I. Klepp, A. Giofré et al., “Virulence factors of the Mycobacterium tuberculosis complex,” Virulence, vol. 4, no. 1, pp. 3–66, 2012.
[3] A. Giofré, E. Infante, D. Aguilar et al., “Mutation in mce operons attenuates Mycobacterium tuberculosis virulence,” Microbes and Infection, vol. 7, no. 3, pp. 325–334, 2005.
[4] A. Williams, A. Davies, P. D. Marsh, M. A. Chambers, and R. G. Hewinson, "Comparison of the protective efficacy of bacille Calmette-Guérin vaccination against aerosol challenge with Mycobacterium tuberculosis and Mycobacterium bovis," Clinical Infectious Diseases, vol. 30, supplement 3, pp. S299–S301, 2000.
[5] M. S. Lever, A. Williams, and A. M. Bennett, “Survival of mycobacterial species in aerosols generated from artificial saliva,” Letters in Applied Microbiology, vol. 31, pp. 238–241, 2000.
[6] S. O. Clark, Y. Hall, D. L. F. Kelly, G. J. Hatch, and A. Williams, “Survival of Mycobacterium tuberculosis during experimental aerosolization and implications for aerosol challenge models,” Journal of Applied Microbiology, vol. 111, no. 2, pp. 350–359, 2011.
[7] J. M. Hartings and C. J. Roy, "The automated bioaerosol exposure system: preclinical platform development and a respiratory dosimetry application with nonhuman primates," Journal of Pharmacological and Toxicological Methods, vol. 49, no. 1, pp. 39–55, 2004.
[8] A. Williams, B. W. James, J. Bacon et al., "An assay to compare the infectivity of Mycobacterium tuberculosis isolates based on aerosol infection of guinea pigs and assessment of bacteriology," Tuberculosis, vol. 85, no. 3, pp. 177–184, 2005.
[9] A. Williams, G. J. Hatch, S. O. Clark et al., "Evaluation of vaccines in the EU TB Vaccine Cluster using a guinea pig aerosol infection model of tuberculosis," Tuberculosis, vol. 85, no. 1, pp. 29–38, 2005.
[10] F. C. Blanco, M. V. Bianco, S. Garbaccio et al., “Mycobacterium bovis Δmce2 double deletion mutant protects cattle against challenge with virulent M. bovis,” Tuberculosis, vol. 93, no. 3, pp. 363–372, 2013.
