Vaccine 29 (2011) 4302–4307

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Vaccination of guinea pigs using *mce* operon mutants of *Mycobacterium tuberculosis*

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ARTICLE INFO

Article history:
Received 17 December 2010
Received in revised form 31 March 2011
Accepted 7 April 2011
Available online 22 April 2011

Keywords:
Tuberculosis
Vaccination
Guinea pig
Attenuated mutants

ABSTRACT

The limited efficacy of the BCG vaccine for tuberculosis, coupled with emerging information suggesting that it is poorly protective against newly emerging strains of *Mycobacterium tuberculosis* such as the W-Beijing isolates, makes it paramount to search for more potent alternatives. One such class of candidates is attenuated mutants derived from *M. tuberculosis* itself. We demonstrate here, in an initial short term assay, that mutants derived from disruption of the *mce* genes of the bacillus were highly protective in guinea pigs exposed by low dose aerosol infection with the virulent W-Beijing isolate SA161. This protection was demonstrated by a significant reduction in the numbers of bacilli harvested from the lungs, and dramatic improvements in lung histopathology.

1. Introduction

Tuberculosis continues to be one of the leading causes of mortality by an infectious disease in the world [1–4]. The deterioration in public health systems in developing countries, and the emergence of multi-drug resistance (MDR) and extensively drug resistant (XDR) isolates leading to forms of tuberculosis that are extremely difficult to treat are major factors, driven by the HIV/AIDS co-pandemic [5–11]. Although chemotherapeutic regimens now exist, they are by necessity both extremely lengthy and expensive, with concomitant problems in terms of compliance and multidrug resistance. As for vaccination strategies, prophylactic vaccination with the attenuated strain *Mycobacterium bovis* BCG remains the only licensed choice, and while it is effective against severe forms of childhood tuberculosis, in adults it has little protective effect [12,13]. For this reason the past two decades have seen a new effort to construct innovative new vaccines for tuberculosis, including a variety of new attenuated live vaccine strains.

The current primary strategy to improve vaccination against tuberculosis is to use recombinant forms of the existing BCG vaccine, alone and in prime boost regimens [14,15]. However, the short-comings of BCG are well known, and this is compounded by new information, including the observation [16] that the newly emerging strains of *Mycobacterium tuberculosis*, including the W-Beijing strains, are mostly of very high virulence, coupled with the observation [17] that these strains induce regulatory T cells capable of interfering with vaccine-induced immunity. This raises the concern therefore that BCG, or rBCG for that matter, will not only be not potent enough to stop these emerging isolates, but may in fact actively select for them [18].

New methods have facilitated genetic manipulation of *M. tuberculosis* [19,20]. These advances, in combination with the availability of the complete sequence of the genome of the bacillus [21] have enabled the study of the contribution of individual genes to virulence. One of the approaches to identify the genes responsible for pathogenicity has been the construction of *M. tuberculosis* mutants, which are then tested for multiplication in the lungs of mouse or guinea pigs. Several studies using this functional genomic approach have reported the development of auxotrophic 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 [13,22–24]. The advantage of using attenuated *M. tuberculosis* strains as vaccine candidates is that only selected genes are targeted, whereas the current vaccine, BCG, lacks >100 genes compared to its parent strain [25].

The 1993 observation that a DNA fragment from *M. tuberculosis* encoding proteins capable of mediating entry into mammalian cells led to the discovery of the *mce* operon [26], 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, as well as the susceptibility of the mouse strain

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doi:10.1016/j.vaccine.2011.04.027

used, in the majority of studies loss of one or more of the *mce* operons usually seems to result in attenuation [27–32]. In contrast, mutation of the gene encoding for *mce1R* (the mce1 operon repressor) leading to increased expression of this operon increased the virulence of the resulting mutant [33].

In this study we investigated the capacity of four such mutants to vaccinate guinea pigs against low dose aerosol infection with a virulent W-Beijing strain of *M. tuberculosis*. All of the mutants tested protected these animals in a short-term challenge assay, and reduced damaging lung pathology compared to unprotected controls. These candidates thus comprise a new set of potential vaccines which should be further tested against the newly emerging high virulence clinical isolates.

2. Materials and methods

2.1. Generation of mutant vaccine strains

Mutant Δmce1, Δmce2 and Δmce3-3 were generated as previously described [26]. Briefly, site-directed mutant strains of *M. tuberculosis* in *mce1*, *mce2* and *mce3* operons were created by two-step mutagenesis using the pPR27 shuttle plasmid, which carries the counterselectable marker *sacB* and a thermosensitive origin of replication. *yrbE1B*, *mce2A* and *mce3A*, were interrupted in independent bacterial clones by insertion of either kanamycin or hygromycin cassette [26].

2.2. Guinea pigs

Female specific pathogen-free albino Hartley-strain outbred guinea pigs of approximately 4-weeks of age were purchased from the Charles River Laboratories (Charles River Breeding Laboratories, Inc., Wilmington, MA). They were allowed to acclimate and reach 500–600 g of weight before use. They were housed in an ABL-III facility and provided commercial chow in stainless-steel feeders and tap water *ad libitum*. Animals were maintained in a temperature- and humidity-controlled environment and exposed to a 12 h light/dark cycle. After challenge animals were monitored using a modified Karnovsky scale in case of stress; no adverse responses were observed over the thirty day challenge period that warranted euthanasia. All protocols were pre-approved by the IACUC at Colorado State University.

2.3. Vaccination and challenge

Animals were vaccinated by the subcutaneous route with 5×10^4 *M. bovis* BCG Pasteur or with an identical dose of each of the *mce* mutants, delivered in 100 μl of sterile saline. They were challenged six weeks later by low dose aerosol exposure to the W-Beijing *M. tuberculosis* strain SA161 [16]. For each aerosol infection, a thawed aliquot of the strain of *M. tuberculosis* was diluted in sterile water up to a total volume of 20 ml (10^6 CFU/ml). Animals are then aerosolized using a Madison infection chamber (University of Wisconsin Machine Shop, Madison, WI) with a starting volume of 10 ml of working stock (nebulization cycle: 5 min; cloud decay cycle: 10 min). Previous analysis of this protocol using magnetic resonance imaging revealed an uptake of approximately 20–30 bacilli into the lungs using this procedure [34].

2.4. Assessment of the bacterial load in target organs

To determine the bacterial load on day 30, the abdominal and thoracic cavities were opened aseptically and the right lung and spleen from individual animals placed into three respective vials filled with 9 ml of sterile saline. Individual tissue samples were homogenized separately using a handheld tissue homogenizer. Serial dilutions of tissue homogenates are then plated onto duplicate Middlebrook 7H11 agar plates and incubated at 37 °C for approximately 21 days. The number of viable *M. tuberculosis* colonies from the appropriate dilution quadrants (i.e., 20 CFU < optimal quadrant CFU count < 200 CFU) from duplicate plates are averaged and expressed as $\log_{10}$ colony forming units (CFU) per total right lung.

To assess the possibility that some mutant bacilli reached the lungs and this then influenced the subsequent measurement of the total lung bacterial load, tissue homogenates were also additionally plated on kanamycin or hygromycin containing agar so that only mutant colonies could grow. We found no evidence however that these bacteria had reached the lungs after subcutaneous inoculation.

2.5. Histopathologic analysis

The left caudal lung lobe from each guinea pig ($n = 5$) was collected at necropsy and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS). Randomly selected tissue sections were embedded in paraffin and cut to 5 μm on a microtome. Tissue sections were mounted on glass slides, deparaffinized and stained with hematoxylin and eosin as previously described [35]. The lung lesion area was quantified relative to normal tissue area using a stereology based method referred to as the Area Fraction Fractionator with the investigator blind to all treatment groups. The lung and lesion area was determined on representative sections. The area of inflammation relative to the normal tissue parenchyma was estimated from representative lung sections evaluated at 20×. A total of 8–12 fields were selected randomly by the computer and a counting frame (2000 μm^2) containing probe points with a grid spacing of 200 μm, was used to define the areas of interest (lesion and lung).

3. Results

3.1. Capacity of Mce mutants to vaccinate guinea pigs against M. tuberculosis

The numbers of CFU cultured from the lungs and spleens of each group is shown in Fig. 1. Guinea pigs vaccinated with BCG were protected by >1-log compared to saline controls ($p < 0.01$), and animals receiving each of the Δ*mce* mutant vaccines had similar levels of protection. In terms of CFUs in the lungs, there was no statistical difference between the protection afforded by BCG or the Δ*mce* mutants.

Reduction in the capacity of the challenge infection to disseminate from the lungs to the spleen is an additional measure of vaccine efficacy. This was substantially prevented by BCG vaccination ($p < 0.01$), with each of the experimental vaccines giving equal or higher protection (in several individual guinea pigs no evidence of dissemination was seen at all).

3.2. Lung pathology

All vaccinated animals exhibited considerably improved histopathology in the lungs compared to the saline controls (Fig. 2). Unprotected controls showed extensive granulomatous inflammation with large primary granulomas exhibiting very extensive caseating central necrosis, as previously noted [16]. Even though BCG vaccination prior to virulent challenge with this virulent W-Beijing strain of *M. tuberculosis* significantly reduced the extent of lung inflammation, it failed to completely abrogate the extent of lesion necrosis which is consistently seen with less virulent challenge strains such as H37Rv. In contrast, vaccination with the Δ*mce* mutants significantly reduced the severity of the inflammatory

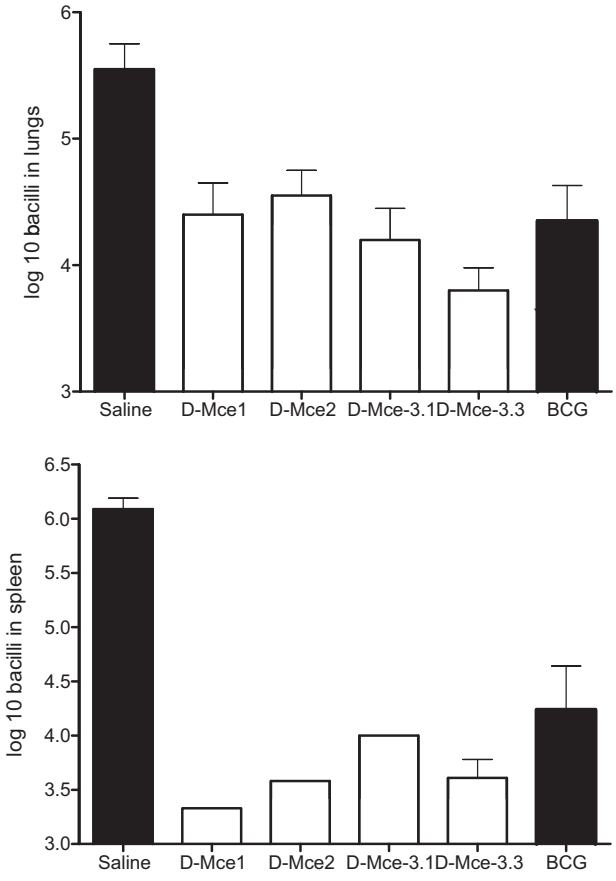

Fig. 1. Day-30 protection assay in guinea pigs immunized with the Δ*mce* mutants or with BCG, and challenged by low dose aerosol with *M. tuberculosis*. The highly virulent W-Beijing strain SA161 was used as the challenge strain. Data is shown as the log10 of the mean lung and spleen burden values ± SEM [$n = 5$]. Significantly higher reduction of lung bacterial burden was observed when guinea pigs were vaccinated with BCG, ΔMce-1, ΔMce3.1 and ΔMce3.3 ($^{**}p < 0.01$) than when vaccinated with ΔMce-2 ($^{*}p < 0.05$ by ANOVA). In the spleen, BCG vaccination significantly reduced bacterial burden ($^{**}p < 0.01$ by student *t*-test).

lesions as well as prevented lesion necrosis. These differences were also reflected by decreases in overall lung consolidation, which in three cases (Δ*mce1* and Δ*mce3*, $p < 0.05$, and Δ*mce3.3*, $p < 0.01$) were even lower than reductions caused by BCG vaccination (Fig. 3).

4. Discussion

The results of this study show that four mutants based on disruption of the *mce* loci of *M. tuberculosis* generated vaccines that were highly protective against a highly virulent W-Beijing clinical isolate in a short term day-30 protection assay. This protection was demonstrated by a significant reduction in the numbers of bacteria that could be recovered from the lungs of the infected guinea pigs, with an equally significant reduction in the capacity of the challenge infection to disseminate to the spleen, and dramatic improvements in histopathology. These observations, coupled with previous observations [13,22–24] of the potency of several different mutants of *M. tuberculosis* as potential vaccines, continue to illustrate the possible usefulness of these approaches in vaccine design.

It is noteworthy that all four *mce* mutants significantly reduced the necrotizing pathology seen in the lungs, and this was particularly evident in guinea pigs vaccinated with the mutant lacking the *mce2* gene. While the calculated lesion to lung ratios and necrosis to lesion ratios in these animals were similar to the others (data not shown), lungs harvested from these animals showed lymphocytic granulomas which were relatively small and which had only minimal necrosis. This is important because prevention of even residual necrosis in this animal model is a key factor in preventing the establishment of persisting bacilli which can eventually relapse [36].

These results reinforce our view [37] that looking at alternatives such as attenuated mutants to replace BCG vaccines is of importance. In this regard, whereas BCG was demonstrated to be protective in this conventional "30-day" assay, we have increasing evidence that the protective activity of BCG is not sustained (Ordway, Orme, manuscript in preparation) and starts to decay 6–8 weeks after the challenge infection. This appears be a direct result of the generation of CD4 Foxp3+ regulatory T cells [17], which are potently induced by W-Beijing strains including SA161 used here, and which we suspect are possibly driven by the inflammation and necrosis such highly virulent infections are capable of causing in the relevant guinea pig model [38]. As noted above, in the present study BCG was initially protective but there was still observable necrosis in lung lesions, whereas there was far less so in the animals given the *mce* mutant vaccines. Whether these vaccines can prevent eventual regulatory T cell induction and hence prevent the loss of protection we are now observing for BCG in longer term studies remains unknown and should be further investigated. Such studies should also include other potent mutants, such as ΔsecA2 and ΔPhoP, in which potent protective effects in animal models have been recently observed [23,39].

Increasing evidence indicates Mce proteins are related to the virulence of the *M. tuberculosis* complex. An earlier study [40] reported that a BCG strain mutated in *mce1* exhibited a reduced ability to invade the non-phagocytic epithelial cell line HeLa. Further studies [28,41] have found that *mce1* disruption provokes attenuation of *M. tuberculosis* after intratracheal or intravenous challenge in the mouse model. Recently, it was shown [42] that mice infected with a *M. tuberculosis* mutant in *fadD5* (*Rv0166*), a gene located within the *mce1* operon, survived longer that those infected with the wild type strain. In contrast, however, a 2003 study [32] that an *mce1* mutant of *M. tuberculosis* killed mice more rapidly than the wild-type strain, possibly a consequence of the infection route. Also, mutation of either *mce2* or *mce3* operons has been shown to attenuate the replication of *M. tuberculosis* in organs of BALBc/mice [28], and it has been recently demonstrated that deletion of *mce2* operon impairs the virulence of *M. tuberculosis* in C57BL/6 mice, in terms of survival and lung pathology [30]. In all these cases the transcription of target genes as well as of their downstream genes in each operon was turned off (by a polar effect). Therefore, while in Δmce3-1 none of the *mce3* genes are expressed, mutations in Δmce3-3 strain would not affect the transcription of both *yrbE3A* and *yrbE3B*. Thus, in consequence, the expression of YrbE3A and B in Δmce3-3 but not in Δmce3-1 could be the reason for the increased protective activity observed above.

Returning to the practical use of such vaccines, it has been recently shown [43] that BCG is poor at generating central memory T cells and that this might be an inherent weakness in terms of its apparently limited protective capacity [37], but whether attenuated forms of *M. tuberculosis* have a similar deficiency is not known. While there is a safety element regarding the use of such mutants in general, this may have to be tempered against the highly virulent and inflammatory nature of the newly emerging clinical isolates [16,44], especially if (as our own preliminary studies show) the protective effect of BCG is only transient. In our hands these newly emerging strains are potent inducers of regulatory T cells in both the mouse and guinea pig models, and preliminary studies in our laboratory indicate that protection mediated by BCG rapidly diminishes at a time that regulatory T cells accumulate in the lungs. This

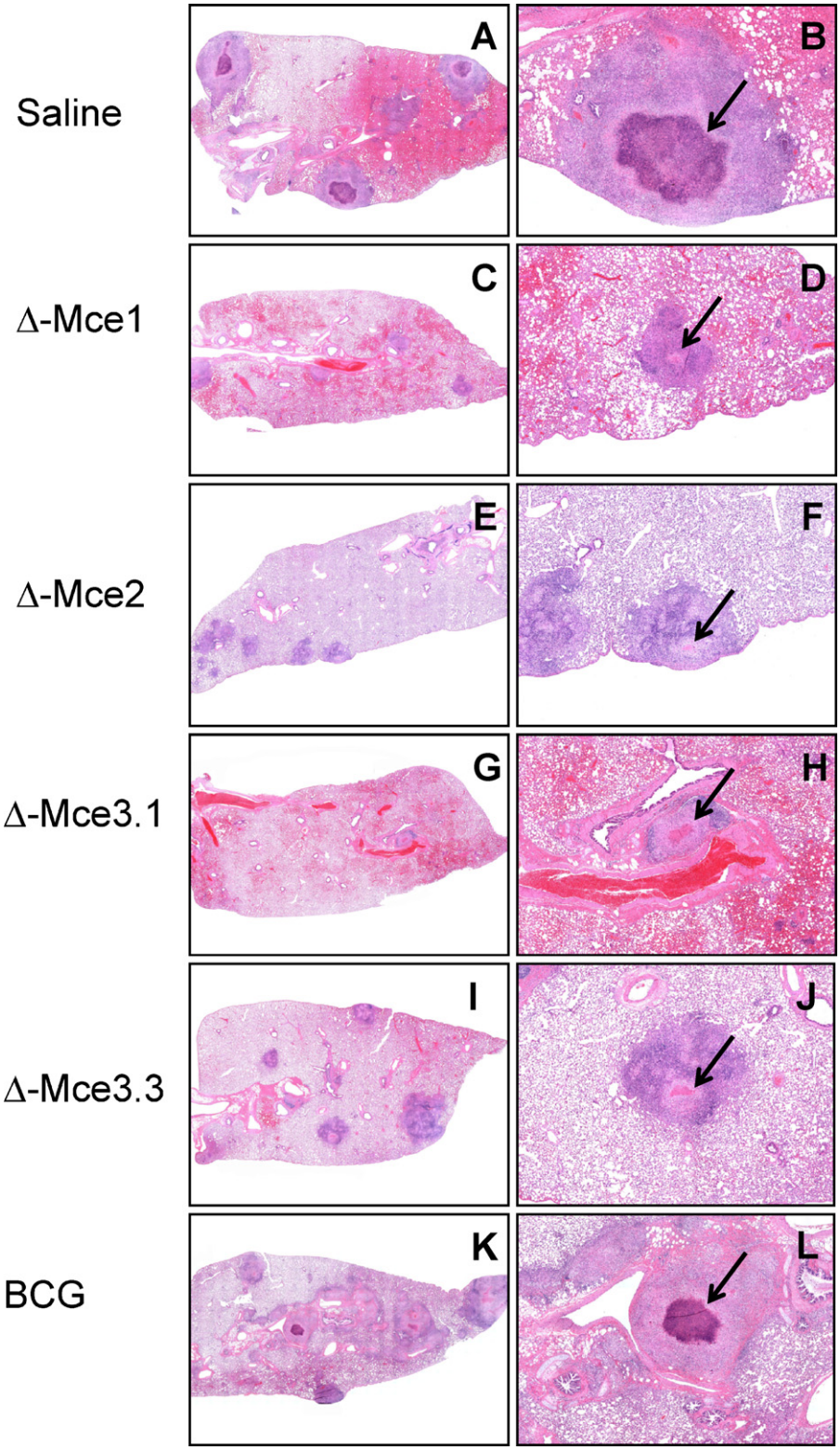

Fig. 2. Vaccination of guinea pigs with Mce vaccines significantly reduces lung pathology after challenge with a highly virulent W-Beijing isolate. Sham vaccination with saline followed by low dose aerosol infection with *M. tuberculosis* SA161 produced multiple primary granulomatous lesions with extensive central necrosis (arrow) (A and B). In contrast, vaccination with, Δ-Mce1 (C and D), Δ-Mce2 (E and F), Δ-Mce3.1 (G and H), or Δ-Mce3.3 (I and J) resulted in better protection characterized by significantly fewer and smaller primary lesions with minimal central necrosis (arrows) that was improved compared to animals vaccinated with BCG (K and L). Hematoxylin and eosin, (A), (C), (E), (G), (I), (K) mag. 2.5×, (B), (D), (F), (H), (J), (L) mag. 100×.

could be a serious impediment to the efficacy of new BCG-based vaccines [37].

To overcome the incomplete protection afforded by BCG numerous vaccine strategies have been developed and tested in experimental vaccine assays. Among them, prime-boost strategies combining single antigens and BCG have shown to significantly improve the protective efficacy of BCG [14,45]. Hence, an attractive alternative to the use of BCG is the development of attenuated

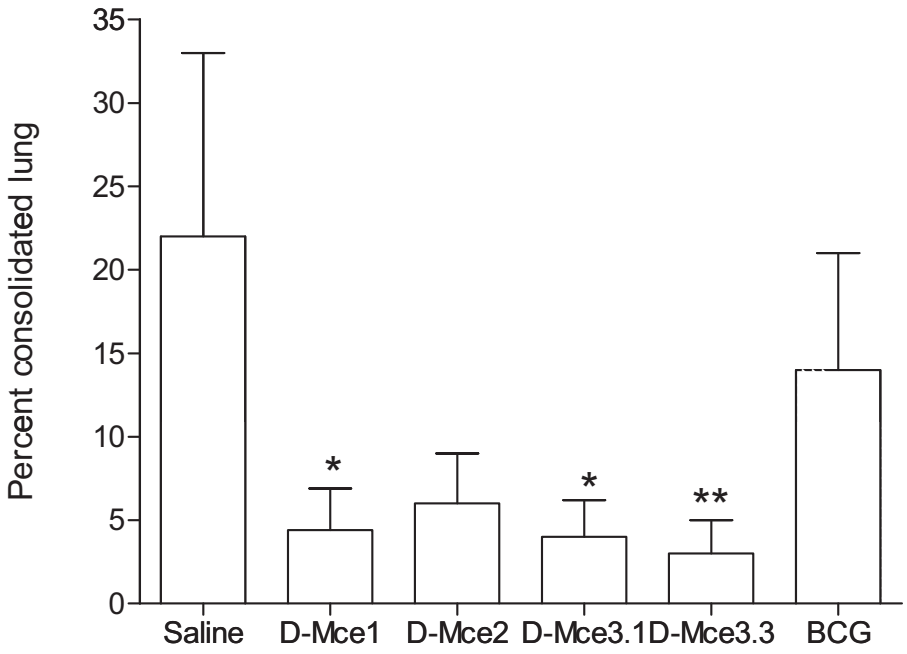

Fig. 3. Percentage degree of consolidated lung in each experimental group. Data shown as mean + SEM ($n = 4–5$ guinea pigs). Three vaccines gave reduced consolidation compared to the BCG control ($^{*}p < 0.05$, $^{**}p < 0.01$, by ANOVA).

M. tuberculosis strains which retain sustained and long-lasting immunity, and which could replace BCG in prime-boost strategies combining live and subunit vaccines. Such strategies could provide a useful alternative to combat the highly virulent clinical strains now emerging. Having said that, a potential drawback to the use of attenuated mutants regards their potential safety; after all, many of those tested to date are still quite potent. It is encouraging therefore to learn that at least one major candidate, the ΔPhoP attenuated mutant, has now undergone comprehensive safety testing in both the mouse and guinea pig models with no evidence of any adverse effects [23,24]. This is of course highly encouraging.

Acknowledgements

This work was supported in part by NIH grant AI-070456 and ANPCyT PICT 2324. We are grateful to Dr Kathy Eisenach for providing strain SA161.

Conflict of interest: None.

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