Immunogenicity and protection induced by a *Mycobacterium tuberculosis* sigE mutant in a BALB/c mouse model of progressive pulmonary tuberculosis.

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**Keywords**: Tuberculosis vaccination, sigE, experimental tuberculosis.

**Abbreviated title**: A new attenuated vaccine against TB

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ABSTRACT

Tuberculosis is still one of the main challenges to human global health, leading to about two million deaths every year. One of the reasons for its success is the lack of efficacy of the widely used vaccine *Mycobacterium bovis* BCG.

In this paper we analyze the potential use of an attenuated mutant of *Mycobacterium tuberculosis* H37Rv lacking the sigma factor $\sigma^E$ as a live vaccine. We demonstrated that BALB/c mice infected by the intratracheal route with this mutant strain, showed significant higher survival and less tissue damage than animals infected with the parental or complemented mutant strains. Although animals infected with the $\text{sigE}$ mutant had low bacillary loads, their lungs showed significantly higher production of the protective factors IFN-\(\gamma\), TNF-\(\alpha\), iNOS and $\beta$-defensins than those of animals infected with the parental or complemented mutant strains. Moreover, we demonstrate that the $\text{sigE}$ mutant, when inoculated subcutaneously, was more attenuated than BCG in immunodeficient nude mice, thus representing a good candidate for a novel attenuated live vaccine strain. Finally, when we used the $\text{sigE}$ mutant as subcutaneous vaccine, it was able to induce a higher level of protection than did BCG with both H37Rv and a highly virulent strain of *M. tuberculosis* (Beijing code 9501000).

Taken together, our findings suggest that the $\text{sigE}$ mutant is a very promising strain for the development of a new vaccine against tuberculosis.
**INTRODUCTION**

Tuberculosis (TB) is still one of the leading causes of mortality throughout the world (10, 30). The HIV/AIDS pandemic, the deterioration in public health systems in developing countries, and the emergence of multi-drug resistance (MDR) forms of TB are important factors contributing to the high toll imposed by this disease on the human population. Prophylactic vaccination with the attenuated strain of *Mycobacterium bovis* Bacille Calmette-Guerin (BCG) is used in most countries. BCG vaccination, even if effective against severe forms of childhood tuberculosis, has a limited efficacy against adult pulmonary disease, the most transmissible form of the infection (11). Hence, new rationally constructed vaccine candidates are required.

*Mycobacterium tuberculosis* is a remarkable pathogen capable of adapting and surviving to various harsh conditions encountered during infection. Such adaptation is mostly due to a complex transcriptional regulatory network able to modulate the expression of its complex genome (37).

Sigma factors bind to the RNA polymerase holoenzyme providing its specificity for particular promoters and play a key role in the regulation of gene expression and adaptation to stress in prokaryotes (5). The *M. tuberculosis* genome encodes for 13 sigma factors, 10 of which belong to the extracytoplasmic function (ECF) subclass (also referred to as group four) (33). Among the mycobacterial sigma factors σE (belonging to the ECF subclass) is probably the best characterized. It is subject to very complex regulation (8), and was clearly shown to be deeply involved in virulence. A mutant in which its structural gene (*sigE*) was disrupted was not only sensitive to various surface-disrupting stresses as the detergent sodium dodecyl-sulphate (SDS), the cationic peptide polymyxin, and the antibiotic vancomycin (12, 26, 31), but was also unable to grow in resting macrophages and dendritic cells, was more sensitive to killing from activated macrophages, and was severely attenuated in mice (3, 14, 27, 29). The σE transcriptome was analyzed by DNA microarrays following SDS-induced surface stress and during macrophage infection (13, 29): interestingly, σE was found to regulate genes involved in mycolic acid biosynthesis and fatty acid degradation, as well as genes involved in membrane proteins quality control and membrane stabilization. Taken together these data suggest that σE is responsible for controlling surface stability and composition following the exposure to damaging environmental conditions.
Finally, recent in-vitro studies comparing the transcriptional response of human and murine macrophages (13) as well as human dendritic cells (14) infected with wild type *M. tuberculosis* strain H37Rv and the *sigE* mutant, revealed that components of the $\sigma^E$ regulon modulate the innate immune system, so that in the *sigE* mutant-infected cells, there was an up-regulation of proteins of the acute phase response, Toll-like receptors 1 and 2, proinflammatory cytokines, chemokines and prostaglandins. Thus, the *sigE* mutant strain stimulates the host immune system during macrophage infection, suggesting that this strain could be an efficient live attenuated vaccine strain.

Rationally attenuated, live replicating mutants of *M. tuberculosis* are potential vaccine candidates (16). The advantage of using attenuated *M. tuberculosis* strains is that they produce a large number of protective antigens, including those that are absent from BCG (4). Thus, vaccination with live attenuated *M. tuberculosis* can induce a stronger and longer immune stimulation, conferring higher levels of protection against TB than BCG (16). In the first part of this paper we describe the survival, lung bacillary loads, histopathology and cytokine profile expression in BALB/c mice after intratracheal infection with the *M. tuberculosis* *sigE* mutant, its parental strain H37Rv, and the *sigE* mutant complemented strains. In the second part we analyzed the potential of the *sigE* mutant as a vaccine. After corroborating its attenuation when administered by the subcutaneous route, and comparing its immunogenicity to that of BCG before challenge, we tested its protective efficacy after challenge with either *M. tuberculosis* H37Rv or one clinical isolate (Beijing strain 9501000) previously shown to be strikingly hypervirulent in BALB/c mice (24). These experiments showed that the *sigE* mutant is able to confer a significantly better protection to challenge with virulent *M. tuberculosis* than BCG.
MATERIALS AND METHODS

Ethics Statement. Animal studies were approved by the Institutional Ethics Committee of the National Institute of Medical Sciences and Nutrition “Salvador Zubirán” in accordance to the guidelines of the Mexican national regulations on Animal Care and Experimentation NOM 062-ZOO-1999.

Growth of bacterial strains. ST28, a sigE mutant and its complemented derivative ST29 were obtained from M. tuberculosis H37Rv as previously described (29). The BCG strain used was M. bovis BCG Phipps. This BCG substrain was the most protective of 10 strains tested in our BALB/c model of progressive pulmonary tuberculosis (6). The Beijing strain code 9501000 was donated by Dr. D. van Soolingen (RIVM, The Netherlands). Strains were grown in Middlebrook 7H9 medium (Difco Laboratories) supplemented with oleic acid, albumin, catalase and dextrose (OADC) (Difco Laboratories). After 1 month of culture, mycobacteria were harvested, adjusted to 2.5x10^5 bacteria in 100 µl phosphate buffered saline (PBS), aliquoted, and maintained at -70ºC until used. Before use, bacterial aliquots were thawed and their viability checked.

Experimental model of progressive pulmonary tuberculosis in BALB/c mice. Virulence (as determined by survival, lung pathology and bacterial load) and immune response induced by each isolate were evaluated in 8 to 10 weeks old male BALB/c mice as previously described (15). Bacilli were growth in liquid culture medium 7H9 and monitored by densitometry. As soon as the culture reached mid-log phase the bacilli were harvested and suspended in phosphate-buffered saline (PBS) containing 0.05% Tween 80 by shaking for 10 minutes with glass beads. The suspension was centrifuged for 1 min at 350g to remove large clumps of bacilli. Then a preliminary bacterial count was achieved by smearing the supernatant at a known ratio of volume to area, and counting 10 random fields after Ziehl-Neelsen staining. The suspension was finally diluted to 2.5x10^5 bacteria in 100µl of PBS and aliquoted at -70°C. Before use bacteria were recounted, and viability checked as described (22). To induce progressive pulmonary tuberculosis, mice were anaesthetized with sevoflurane and inoculated intratracheally with 2.5 x 10^5 CFU of M. tuberculosis H37Rv, the sigE mutant or the sigE complemented strain suspended in 100 µl PBS (15). After animal infection, the remnant of the bacterial inoculum was plated to confirm the number of cfu administrated to the animals.
Infected mice were kept in a vertical position until the effect of anaesthesia passed. Animals were maintained in groups of five in cages fitted with micro-isolators connected to negative pressure. Twenty mice from each group were left undisturbed to record survival from day 8 up to day 120 after infection. Six animals from each group were sacrificed by exsanguinations at 1, 3, 7, 14, 21, 28, 60 and 120 days after infection. One lung lobe, right or left, was perfused with 10% formaldehyde, dissolved in PBS and prepared for histopathological studies. The other lobe was snap-frozen in liquid nitrogen, and then stored at -70°C for microbiological and immunological analysis (15). All procedures were performed in a laminar flow cabinet in a biosafety level III facility.

**Preparation of lung tissue for histology and automated morphometry.** One lobe of the lung was fixed by intratracheal perfusion with 10% formaldehyde for 24 hrs, then sectioned through the hilus and embedded in paraffin. Sections, 5µm thick, were stained with hematoxylin-eosin for the histological-morphometric analysis. The percentage of the pulmonary area affected by pneumonia was determined using an automated image analyzer (Q Win Leica, Milton Keynes) (15).

**Determination of colony-forming units (CFU) in infected lungs.** Right or left lungs from four mice at each time point, in two separate experiments, were used for colony counting. Lungs were homogenized with a Polytron (Kinematica, Luzern, Switzerland) in sterile 50 ml tubes containing 3 ml of isotonic saline. Four dilutions of each homogenate were spread onto duplicate plates containing Bacto Middlebrook 7H10 agar (Difco Labs, Detroit MI, USA) enriched with (OADC). Plates were incubated for 21 days prior to determination of colony forming units (CFU) (15).

**Real time PCR analysis of cytokines in lung homogenate.** Left or right lung lobes from three different mice per group in two different experiments were used to isolate mRNA using the RNeasy Mini Kit (Qiagen), according to recommendations of the manufacturer. Quality and quantity of RNA were evaluated through spectrophotometry (260/280) and on agarose gels. Reverse transcription of the mRNA was performed using 5 µg RNA, oligo-dT, and the Omniscript kit (Qiagen, Inc). Real-time PCR was performed using the 7500 real time PCR system (Applied Biosystems, USA) and Quantitect SYBR Green Mastermix kit (Qiagen). Standard curves of quantified and diluted PCR product, as well as negative controls, were included in each PCR run. Specific primers for genes encoding glyceraldehyde-3-phosphate dehydrogenase (G3PDH), TNF-α, IFN-γ, IL-4, IL-10, β-defensin 3 (βD3), and β-defensin 4 (βD4) were designed using the program Primer Express (Applied Biosystems, USA) (Tab. 1):
Cycling conditions used were: initial denaturation at 95°C for 15 min, followed by 40 cycles at 95°C for 20 sec, 60°C for 20 sec, 72°C for 34 sec. Quantities of the specific mRNA in the sample were measured according to the corresponding gene specific standard. The mRNA encoding G3PDH was used as internal invariant control to normalize the expression of the cytokine-expressing genes. Data were shown as copies of cytokine-specific mRNA/10^6 copies of G3PDH-specific mRNA (1).

Comparison of immunogenicity of BCG and sigE mutant vaccinated mice before challenge. To study bacterial growth and ability to disseminate, we determined CFU in different organs after subcutaneous vaccination (1).

Groups of four BALB/c mice were vaccinated by inoculating the best protective dose of live bacilli (8000 cells, determined as described above, data not shown) subcutaneously at the base of the tail. After animal vaccination, aliquots of the bacterial inoculum were plated to confirm the number of cfu administrated to the animals.

Animals were killed at 15, 30 and 60 days post-vaccination. The inguinal lymph nodes, spleen, lungs, and the subcutaneous tissue at the site of vaccination (base of the tail) were immediately dissected and homogenized for determination of bacillary loads by CFU quantification following the same procedure described above.

Another group of four vaccinated BALB/c mice per time point was used to determine immunogenicity by comparing the production of IFN-γ by cell suspensions from inguinal lymph nodes, spleen and lungs after stimulation with mycobacterial culture filtrate antigens (CFA), or the immunodominant recombinant antigens ESAT-6, Hsp65 and Ag85 (1). After killing the mice, spleen, inguinal lymph nodes and lungs were immediately removed, placed in 2 ml of RPMI medium containing 0.5 mg/ml collagenase type 2 (Worthington, NJ, USA), and incubated 1 hour at 37°C. Samples were then passed through a 70-µm cell sieve, crushed with a syringe plunger, and rinsed with the medium. Cells were centrifuged at 1500 rpm for 5 min and the supernatant was removed; red cells were eliminated with a lysis buffer. After washing, cells were resuspended in RPMI medium supplemented with 2 mM L-glutamine, 100 U of penicillin per ml, 1 µg of streptomycin per ml (Sigma), and 10% fetal calf serum. Cultures for cytokine production (10^6 cells in 1 ml of culture medium) were performed in flat-bottomed 24-well plates without and with mycobacterial antigens (CFA, ESAT-6, Hsp65, and Ag85). After 3 days of antigenic stimulation, cells were centrifuged and the supernatant used for IFN-γ quantification through a commercial ELISA test kit (Pharmingen, San Diego, CA, USA). Preliminary dose-response curve experiments
showed that the best antigen concentration was 5 µg during 3 days of culture stimulation (data not shown).

**Nude mice infections.** Groups of 20 nude mice were vaccinated subcutaneously at the base of the tail with one dose of 8000 live sigE mutant or BCG bacilli. After animal infection, the remnant of the bacterial inoculum was plated to confirm the number of cfu administrated to the animals (1)

**Evaluation of protection against M. tuberculosis H37Rv and high virulent Beijing-strain in BALB/c mice vaccinated with the sigE mutant or BCG.** Two separate experiments were performed using 10 mice for each of four experimental groups. Animals were vaccinated by inoculating the best protective dose of live bacilli (8000 cells, data not shown) subcutaneously at the base of the tail. At 60 days post-vaccination, the first group of 10 mice was challenged through the intra-tracheal route with 2.5 x 10⁵ CFU of *M. tuberculosis* H37Rv, while the second group with the same number of animals was challenged by the same route and dose with the highly virulent Beijing-strain code 9501000. The third and fourth groups corresponded to control animals which were not vaccinated and were intratracheal infected with the same dose of either H37Rv or the Beijing strain. Three mice/group were euthanized and their lung homogenates were used to determine the infecting dose which resulted around 2.1-2.3 x 10⁴ CFU (not shown). After 2 and 4 months post-challenge, levels of protection were determined by the quantification of CFU in lung homogenates, following the same procedure described above, and by automated morphometry, measuring the lung surface affected by pneumonia. Ten more animals per group were left untouched and deaths were recorded to construct survival curves.

**Statistical analysis.** Statistical analysis for survival curves was performed using Kaplan-Meier plots and Log Rank tests. Student’s t test was used to determine statistical significance of CFU, histopathology and cytokine expression, *P* < 0.05 was considered as significant.
RESULTS

Characterization of the sigE mutant pathogenicity after intra-tracheal administration. In order to characterize the sigE mutant attenuation in our model, groups of BALB/c mice (70 per group) were infected intratracheally with $2.5 \times 10^5$ CFU of H37Rv, the sigE mutant, or its complemented strain. All the animals infected with the sigE mutant survived after four months of infection. In contrast, mice inoculated with the complemented or parental strain started to die at three weeks post-infection and all had died by 8 weeks (Fig 1A). These survival rates well correlated with the CFU in lung homogenates. During the first and second week of infection, similar numbers of CFU were detected in the three groups, whereas after day 21 and 28 post-infection significantly lower bacterial loads were found in mice infected with the sigE mutant, compared to those detected in animals infected with the parental or complemented strains (Fig 1B). At day 60 and 120, animals infected with the mutant strain still showed a low bacterial burden, while the other animals were dead.

The histopathological analysis showed inflammatory infiltrate predominantly constituted by lymphocytes and activated macrophages (large cells with compact cytoplasm and nucleus with apparent nucleoli) in the alveolar-capillary interstitium and around small blood vessels and bronchial walls after one and two weeks of the infection with either of the strains, being higher in animals infected with the sigE mutant which showed well formed granulomas after 7 days of infection, while the mice infected with the parental or complemented strains showed granulomas after two weeks of infection. After 28 days post-infection only H37Rv induced a significant pneumonia involving about 30% of the lung surface. By contrast, in mice infected with the sigE mutant, well formed granulomas and comparable pneumonia appeared only after 60 and 120 days post-infection, when the animals infected with H37Rv or the complemented strain were all dead (data not shown).

Evaluation of cytokine, iNOS and β-defensines expression during infection. The amount of mRNA specific for various cytokines, iNOS, and β-defensins 3 and 4 were evaluated during the infection with the three different strains. Although the lungs of mice infected with the sigE mutant showed significant lower bacillary loads and inflammation than animals infected with the parental or complemented strains, they showed a significant higher and constant expression of genes encoding IFN-γ, TNF-α,
and β-defensin 3 (Fig. 2), as well as a progressive induction of iNOS expression in late time points (Fig. 2). Also expression of IL-10 was higher in sigE infected mice, but only from 21 and 28 days after the infection (Fig. 2). The only cytokine shown to be expressed at a significant lower level in the sigE infected animals was IL-4 (Fig. 2).

Comparison of sigE mutant and BCG attenuation in mice. In order to compare the virulence of the sigE mutant to that of BCG, groups of BALB/c mice (12 per group) were inoculated subcutaneously with 8000 CFU of either of these two bacterial strains. Two weeks after inoculum, animals which received the sigE mutant showed a significant two-fold higher bacterial load at the inoculation site and in the lungs. However, at days 30 and 60 post-vaccination, both groups of vaccinated animals showed similar bacillary loads in the inoculation site, inguinal lymph nodes, spleen and lungs (Fig 3), suggesting that the sigE mutant is not more virulent than BCG in mice. To further investigate the virulent potential of the sigE mutant strain compared to that of BCG, we compared the survival rate of nude mice (20 per group) inoculated subcutaneously with 8000 CFU of either of the two bacterial strains. Results, shown in Figure 4 show that, even if no significative difference in the 50% survival time point was found between the two groups, at the end of the experiment there was a significant difference among the survival between the two groups suggesting that the sigE mutant is more attenuated than BCG in these immunodeficient animals.

Comparison of sigE mutant and BCG immunogenicity following vaccination. In order to compare the efficiency of cellular immunity activation induced by sigE mutant or BCG vaccination, we quantified by ELISA the IFN-γ production in cell suspensions collected from spleen, lung and inguinal lymph nodes collected at different time points after vaccination and stimulated with mycobacterial antigens. Figure 5 shows that spleen and lung cells from animals vaccinated with the sigE mutant stimulated with culture filtrate antigens (CFA) or with the other recombinant antigens produced significant higher levels of IFN-γ than BCG-vaccinated mice at day 60 post-vaccination. Since BCG lacks the ESAT-6 structural gene, animals vaccinated with this strain did not produce or secrete any significant amount of IFN-γ after stimulation with this antigen.

Comparative protection against M. tuberculosis H37Rv or Beijing-9501000 in BALB/c mice vaccinated with the sigE mutant or BCG. In order to compare the level of protection induced by BCG or the sigE mutant, groups of BALB/c mice (40 per
group for 2 separate experiments) were vaccinated subcutaneously in the base of the tail with 8000 CFU of either strain. At 60 days post-vaccination, mice were challenged intratracheally with $2.5 \times 10^5$ CFU of *M. tuberculosis* H37Rv. Ten mice were euthanized at 60 or 120 days post-challenge. Levels of protection were determined by survival rates, quantification of CFU recovered from the lungs, and the extension of tissue damage evaluating the percentage of the lung surface affected by pneumonia in both time points.

After four months post-challenge 98% of the mice vaccinated with the *sigE* mutant were still alive, while 20% of BCG vaccinated mice had died. All non-vaccinated controls died after 11 weeks of infection (Fig.6A, left panel). These results well correlated with lung bacillary loads and histopathology, showing significant less CFU and pneumonia in mice vaccinated with the *sigE* mutant than in BCG vaccinated or control non-vaccinated animals. In particular, *sigE* vaccinated mice showed at 60 and 120 days post-infection a reduction of lung cfu of 1.1 and 1.0 $\log_{10}$ with respect to BCG vaccinated mice, and a reduction of 1.4 $\log_{10}$ with respect with non vaccinated controls at day 60 post-infection (Fig 6B, and C, left panel).

In a second vaccination experiments, animals vaccinated following the same protocol were challenged with the highly virulent *M. tuberculosis* strain Beijing 9501000. Non-vaccinated animals started to die after four weeks from the challenge, and after 6 weeks all were dead. Mice vaccinated with BCG showed a 30% survival after 4 months post-challenge, whereas animals vaccinated with the *sigE* mutant exhibited a significantly higher survival of 80% (Fig 6A, right panel). These results were in agreement with lung CFU determinations (Fig 6B, right panel): after 60 days from the infection mice vaccinated with the *sigE* mutant had a lung bacillary load 0.72 $\log_{10}$ lower than those vaccinated with BCG, while after 120 days the reduction was of 0.79 $\log_{10}$ (Fig 6B, right panel). After 60 days from the challenge, *sigE* vaccinated animals showed a higher percentage of lung surface affected by pneumonia than BCG vaccinated mice, suggesting a more rapid and higher expression of proinflammatory cytokines like IFN-$\gamma$ and TNF-$\alpha$, in *sigE* mutant vaccinated animals which could correlate with better protection. However, after 120 days from the challenge this difference disappeared (Fig 6C, right panel).
DISCUSSION

During infection, bacteria confront different environments determined by the site in which the pathogen resides and the level of activation of the host immune response. To survive and grow, the pathogen must be able to adapt to these different milieus. Most bacterial adaptive mechanisms are based on the regulation of gene expression, which consequently plays a very important role in bacterial pathogenesis (37). Examples of this regulation are the two-component regulatory systems like PhoP-PhoQ (38), and $\sigma$ factors (28).

$\sigma^E$, a member of the ECF subclass of sigma factors, is induced after exposure to different stress conditions, such as heat shock, SDS-mediated cell surface stress, vancomycin, oxidative stress, and alkaline pH, and during growth in human macrophages (33). Its regulon includes several genes involved in stress response and surface biology, as mycolic acid biosynthesis, fatty acids degradation, membrane proteins quality control and membrane stabilization. (33). In a previous study, we demonstrated that the $\text{sigE}$ mutant was attenuated in immunodeficient SCID and immunocompetent BALB/c mice after intravenous infection (27). The aims of the present study were to further characterize its pathogenicity and immunogenicity in BALB/c mice after infection by the intratracheal route, and then to evaluate the potentiality of this mutant as an attenuated vaccine. Our BALB/c mouse model of progressive pulmonary tuberculosis is suitable to determine the virulence and immune response induced by mutant mycobacteria, since it is based on respiratory infection, which is the usual infection route in humans. Moreover, in this model the rate of bacterial multiplication in the lungs well correlates with the extent of tissue damage (pneumonia) and mortality, and the infection is successfully controlled as long as a strong Th1 cell response is sustained (18-20), in agreement with previous evidence on the protective role of Th1 cell-cytokines against mycobacterial infection (7).

Our results confirmed that the $\text{sigE}$ mutant is highly attenuated, permitting complete survival of the infected animals after four months of infection, with significant lower bacillary loads and tissue damage than animals infected with the parental and complemented strains. Despite the observation that lungs of mice infected with the $\text{sigE}$ mutant had a lower bacterial burden and inflammation, they exhibited significant higher expression of IFN-$\gamma$ and TNF-$\alpha$ than the lungs of mice infected with the parental or
complemented strains, suggesting that the \textit{sigE} mutant elicits a stronger immune response. Moreover, the fact that increased level of IFN-\(\gamma\) and TNF-\(\alpha\) were detectable already after 1 day from infection, underscores the ability of the \textit{sigE} mutant to induce very rapidly the secretion of these cytokines. These results are in agreement with recent in-vitro observations of macrophages infected with the same mutant (13). These studies showed that in comparison with resting macrophages infected with the parental strain H37Rv, \textit{sigE} mutant-infected cells exhibited higher expression of the transcriptional factor T-bet and, in consequence, more IFN-\(\gamma\) production. Moreover, IFN-\(\gamma\)-activated macrophages infected in vitro with the mutant strain induced high expression of TNF-\(\alpha\) (13), which could explain the reason for the high induction of iNOS expression that we detected in the \textit{sigE} mutant infected lungs. Interestingly, the lungs of mice infected with the \textit{sigE} mutant showed, during the late stage of infection, higher expression of IL-10, an antinflammatory cytokine that may limit migration of lymphocytes and reduce tissue damage, but that under certain conditions can also exert stimulatory effects on CD4\(_\text{-}\) and CD8\(_\text{-}\) T cells, leading to increased IFN-\(\gamma\) production (23, 25). This finding is in perfect agreement with the high production of IL-10 that we previously observed in human dendritic cells infected in-vitro with the \textit{sigE} mutant (14).

Another interesting observation was the increased expression of \(\beta\)-defensins 3 and 4 in the lungs of mice infected with the \textit{sigE} mutant. These molecules are cationic natural antimicrobial peptides that can kill microbes, and some of them have chemotactic activities on immune cells (9). We have previously shown in this animal model of tuberculosis, a rapid and high expression of \(\beta\)-defensins 3 and 4 during the phase of efficient control of bacillary replication (32). This finding was in perfect agreement with the observation that macrophages infected in-vitro with the \textit{sigE} mutant up-regulates genes encoding Toll-like receptors 1 and 2 and \(\beta\)-defensins (13). Finally, we showed that mice infected with the \textit{sigE} mutant produced significatively less IL-4 than those infected with the wild type parental strain. This is of extreme interest since in our model of infection, induction of IL-4 production correlates with exacerbation of the disease and failure of the immune system to control bacterial replication (17, 36). Thus, the predominant Th-1 response plus the high expression of \(\beta\)-defensins in mice infected with the \textit{sigE} mutant could be the basis of its attenuation allowing the 100% survival in association with very low CFU and tissue damage. It is worth noting that \textit{sigE} complemented strain-infected animals produced IL-4 at levels higher than animals infected with H37Rv: this might be explained by preliminary unpublished data.
suggesting that in the complemented strain $\text{sigE}$ level of expression is higher than in the wt strain.

These observations justify the hypothesis that the $\text{sigE}$ mutant could have a strong potential as a novel attenuated vaccine, since the response to its infection fits well into the proposition that the aim of a “classical” vaccine is to mimic natural infection as closely as possible inducing a strong immune protective response without causing extensive disease (39). In addition, the lack of a Th-2 response in the presence of a strong Th-1 response is considered one of the essential characteristics for a new antitubercular vaccine (34-36). Moreover, the $\text{sigE}$ mutant can be considered a good vaccine candidate since it is highly attenuated in SCID mice (27), and at the latest time points produces a significant lower mortality than BCG in nude mice. This is of particular importance, since one of the problems of BCG is that it can cause disease in immuno-compromised patients, so a new attenuated vaccine strain should be more attenuated than BCG in this kind of patients. Finally, another promising observation was that after vaccination and before challenge, spleen and lung cell suspensions stimulated with mycobacterial antigens from mice vaccinated with the $\text{sigE}$ mutant were more efficient in the production of IFN-$\gamma$ than those from animals vaccinated with BCG. Taken together these observations suggest that the $\text{sigE}$ mutant is safer and more immunogenic than BCG.

Beside the down-regulation of the genes in the $\sigma^E$ regulon, some of which are involved in surface biology, $\sigma^E$ absence has a pleiotropic effect on the bacterial surface, as demonstrated by the transcriptional profile of the $\text{sigE}$ mutant after in-vitro macrophage infection, showing the induction of genes related to cell wall structure and protein secretion (13). Thus, the $\text{sigE}$ mutant might have cell envelope defects resulting in both its attenuation and its high immunogenicity. We are currently comparing the composition of the cell envelope and secretome of the $\text{sigE}$ mutant and wild type parental strain H37Rv in order to identify differences that could be the basis of their different pathogenic and immunogenic behaviour.

Several mycobacterial mutants have already been demonstrated to have good potential as new efficient vaccine (reviewed in 10), and three of them have been analyzed using the model of infection used in this paper: i) a mutant lacking $\text{phoP}$, which was able to induce similar protection to that of BCG (1); ii) a mutant lacking $\text{fadD26}$ (which lacks the cell wall lipid complex phthiocerol dimycocerosate), which conferred 70% survival after four months of challenge with the highly virulent strain Beijing 9501000, but
showed only a partial attenuation (21); iii) a mutant lacking the mammalian cell entry gene 2 (mce2), which was severely attenuated and induced a 72% survival after four months of challenge with the highly virulent strain Beijing 9501000 (2). We show here that the sigE mutant is as attenuated as the mce2 mutant, but induced better protection, allowing 80% mice survival after four months of challenge with strain Beijing 9501000. Interestingly, mice infected with the mce2 mutant, in contrast to those infected with the sigE mutant, produced significantly less IFN-γ than those infected with the wild type strain and did not produce more TNF-α. Thus, the sigE mutant is until now the best vaccine candidate tested in this experimental mouse model.

In our experiments the level of BCG-induced protection was lower than that usually provided by this vaccine. This could be explained by the fact that the mice used in this study received Mexico City autoclaved, but not filtered, tap water, known to contain high loads of environmental mycobacteria. Pre-exposure to these environmental mycobacteria probably resulted in a basal level of immunization against mycobacterial antigens, which have been hypothesized to cause poor responsiveness to BCG vaccination (34, 36). Further experiments in other animal models in which the protective potential of BCG is higher than that obtainable in our model, are needed to confirm the superiority of the protective potential of the sigE mutant with respect to BCG.

We conclude that these results encourage further studies of the sigE mutant as a potential vaccine strain; for this purpose, the construction of a double mutant in order to create a more attenuated and highly immunogenic mutant or the over expression of protective antigens in this strain could represent valuable strategies for further developments.
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REFERENCES


Table 1. Primers used for quantitative RT-PCR determinations

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<th>Target gene</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
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<td>G3PDH</td>
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Figure Legends

FIG. 1. Pathogenicity of the sigE mutant after intratracheal inoculation. (A) Survival of BALB/c mice (20 mice per strain) infected by intratracheal injection of *M. tuberculosis* H37Rv, sigE mutant and complemented strain. (B) Lung bacterial burden in mice infected with *M. tuberculosis* H37Rv, sigE mutant and complemented mutant strain. Mice were sacrificed after 1, 3, 7, 14, 21, 28, 60 and 120 days post-infection.

FIG. 2. Quantitative expression of mRNA was determined by real time PCR in lungs from mice infected with sigE mutant, H37Rv or complemented strain. Data are expressed as means and standard deviation from four different animals at each time point. Asterisks represent statistical significance ($p < 0.05$) when compared with H37Rv infected mice. No data at day 120 post-infection is presented for H37Rv and complemented mutant strain infected mice because no surviving animals were available in these experiments.

FIG 3. Bacillary loads at the site of vaccination (subcutaneous tissue base of the tail), inguinal lymph nodes, spleen, and lungs from BALB/c mice inoculated 8000 cfu of BCG (white bars) or the sigE mutant (gray bars) at different time points before the challenge. Bars represent the means and standard deviation from four different animals at each time point in two separate experiments. Asterisks represent statistical significance ($p < 0.05$) among the indicated groups.

FIG. 4. Survival of nude mice (20 mice per strain) vaccinated by the subcutaneous route in the base of the tail (8000 cfu) with BCG (black triangles) or sigE mutant (white squares). Asterisks represent statistical significance ($p < 0.05$).

FIG. 5. IFN-γ quantification by ELISA. IFN-γ was quantified in cell suspension supernatants from inguinal lymph nodes, lungs and spleen of BCG or sigE mutant vaccinated mice after stimulation with culture filtrate mycobacterial antigens (CFA), and the immunodominant recombinant antigens ESAT-6, Hsp65 and Ag85. Bars
represent the means and standard deviation from four different animals at each time point. Asterisks represent statistical significance ($p < 0.05$).

FIG. 6. Survival, lung bacillary loads, and histopathology after intra-tracheal challenge with H37Rv (left panel) or Beijing 9501000 (right panel) in BALB/c mice vaccinated with the sigE mutant or BCG, and in control non-vaccinated animals (NVA). (A) Survival of the different groups of BALB/c mice (20 mice per strain) challenged with the indicated strains. (B) Lung bacillary loads in the different groups of BALB/c mice challenged with the indicated strains after 60 and 120 days from the challenge. (C) Percentage of lung surface affected by pneumonia determined by automated morphometry. The results are expressed as the mean ± standard deviations in four mice. Asterisks represent statistical significance ($p < 0.005$) between the represented groups. No data at 2 and 4 months post-challenge are presented for the control non-vaccinated animals challenged with the Beijing strain, and at 4 months post-challenge for the control non-vaccinated animals challenged with H37Rv, since no surviving animals were available.
Figure 1

A

CFU x10^6/Lung

% survival

0 20 40 60 80 100 120

0 1 2 3 4

1 3 7 14 21 28 60 120

* *

B

H37Rv

sigE mutant

sigE compl.

H37Rv

sigE mutant

sigE compl.

0 20 40 60

% survival

100

0 1 2 3 4

1 3 7 14 21 28 60 120

* *
Figure 2
Figure 3
Figure 4
Figure 5

Spleen

Lung

Lymph nodes
**Figure 6**

H37Rv

A. % survival

Days post-infection

B. Log10 CFU/lung

Day 60
Day 120

Beijing

C. % of pneumonia

Day 60
Day 120

sigE
BCG
NVA