Immunogenicity and Protection Induced by a Mycobacterium tuberculosis sigE Mutant in a BALB/c Mouse Model of Progressive Pulmonary Tuberculosis

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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 multidrug-resistant (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-Guérin (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 to and surviving various harsh conditions encountered during infection. Such adaptation is due mostly 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 various environmental conditions. 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 various harsh conditions encountered during infection. Such adaptation is due mostly to a complex transcriptional regulatory network able to modulate the expression of its complex genome (37).

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absent from BCG (4). Thus, vaccination with live attenuated
M. tuberculosis can induce a stronger and longer immune stimu-
lation, conferring higher levels of protection against TB than
BCG (16). In the first part of this article, we describe the
survival, lung bacillary loads, histopathology, and cytokine pro-
file expression in BALB/c mice after intratracheal infection with
the M. tuberculosis sigE mutant, its parental strain, H37Rv, and the sigE mutant complemented strain. In the sec-
ond part, we analyze the potential of the sigE mutant as a vac-
cine. After corroborating its attenuation when administered by
the subcutaneous route and comparing its immunogenicity
to that of BCG before challenge, we tested its protective effi-
cacy 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 experi-
ments showed that the sigE mutant is able to confer a signifi-
cantly better protection against challenge with virulent M. tu-
berculosis 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 with 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 de-
scribed (29). The BCG strain used was M. bovis BCG Phipps. This BCG strain was the most protective of 10 strains tested in our BALB/c model of progressive
pulmonary tuberculosis (6). The Beijing strain 9501000 was donated by D. van Soodeling (RIVM, Netherlands). Strains were grown in Middlebrook 7H9 me-
dium (Difco Laboratories) supplemented with oleic acid, albumin, catalase, and dextrose (OADC) (Difco Laboratories). After 1 month of culture, mycobacteria were harvested, adjusted to 2.5 \( \times 10^8 \) bacteria in 100 \( \mu \)l phosphate-buffered saline (PBS), aliquoted, and maintained at −70°C until used. Before use, bac-
terial aliquots were thawed and their viability was checked.

**Experimental model of progressive pulmonary tuberculosis in BALB/c mice.** Virulence (as determined by survival, lung pathology, and bacterial load) and the immune response induced by each isolate were evaluated in 8- to 10-week-old male BALB/c mice as previously described (15). Bacilli were grown 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 PBS con-
taining 0.05% Tween 80 by shaking for 10 min with glass beads. The suspension was centrifuged for 1 min at 350 \( \times g \) 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 2.5 \( \times 10^5 \) bacteria in 100 \( \mu \)l of PBS and aliquoted at −70°C. Before use, bacteria were recounted and viability was checked as described previously (22). To induce progressive pulmonary tuber-
culosis, mice were anesthetized with sevoflurane and inoculated intratracheally with 2.5 \( \times 10^5 \) CFU of M. tuberculosis H37Rv, the sigE mutant, or the sigE complemented strain suspended in 100 \( \mu \)l 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 anesthesia passed. Animals were maintained in groups of five in cages fitted with microiso-
lators 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 h and then sectioned through the hilus and embedded in paraffin. Sections 5 \( \mu \)m thick were stained with hematoxylin-cosin for the histological-morphometric analysis. The percentage of the pulmonary area affected by pneumonia was determined using an automated image analyzer (Q Win Leica; Leica, Milton Keynes, United Kingdom) (15).

**Determination of 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 Polytrom homogenizer (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 Baclt Middlebrook 7H10 agar (Difco Labs, Detroit, MI) enriched with OADC. Plates were incubated for 21 days prior to determination of 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 RNasey minikit (Qiagen), according to the recom-
mandations 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 \( \mu \)g RNA, oligo(dT), and the Omniscript kit (Qiagen, Inc.). Real-time PCR was carried out using the 7500 real time PCR system (Applied Biosystems) and Quantitect SYBR green Mastermix kit (Qia-
gen). 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), tumor necrosis factor alpha (TNF-\( \alpha \)), gamma interferon (IFN-\( \gamma \)), interleukin 4 (IL-4), IL-10, \( p \)-defensin 3 (\( \beta \)D3), and \( p \)-defensin 4 (\( \beta \)D4) were designed using the program Primer Express (Applied Biosystems) (Table 1).

**TABLE 1. Primers used for quantitative RT-PCR determinations**

<table>
<thead>
<tr>
<th>Product of target gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3PDH</td>
<td>CATGTGGAAAGGGCTCATGA</td>
</tr>
<tr>
<td>TGF-beta</td>
<td>TGGGACATGAAATATCTCGAG</td>
</tr>
<tr>
<td>IL4</td>
<td>CGTCCTCACAGCAACCGGAGA</td>
</tr>
<tr>
<td>IL-10</td>
<td>AAAGGACTGACGACATAGGC</td>
</tr>
<tr>
<td>IFN-gamma</td>
<td>TCTGGTTCATTTCTCTGGTG</td>
</tr>
<tr>
<td>( \beta )D3</td>
<td>TGGTGTTCATTTCTCTGGTG</td>
</tr>
<tr>
<td>( \beta )D4</td>
<td>GAAAGGCTATGCAATGGAC</td>
</tr>
<tr>
<td></td>
<td>GCCCGAGAAAGCCTCTTG</td>
</tr>
</tbody>
</table>

**Comparison of immunogenicity with BCG- and sigE-mutant-vaccinated mice before challenge.** To study bacterial growth and ability to disseminate, we deter-
mained CFU in different organs after subcutaneous vaccination (1). Groups of four BALB/c mice were vaccinated by inoculating the best protec-
tive dose of live bacilli (8,000 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 postvaccination. The inguinal lymph nodes, spleen, lungs, and 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 mice were killed, 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), and incubated for 1 h 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 1,500 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 (107 cells in 1 ml of culture medium) were performed in 12-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 was used for IFN-γ quantification through a commercial enzyme-linked immunosorbent assay (ELISA) test kit (Pharmingen, San Diego, CA). Preliminary dose-response curve experiments showed that the best antigen concentration was 5 μg during 3 days of culture stimulation (data not shown).

**Infection of nude mice.** Groups of 20 nude mice were vaccinated subcutaneously at the base of the tail with one dose of 8,000 live sigE mutant or BCG bacilli. After animal infection, the remnant of the bacterial inoculum was plated to confirm the number of CFU administered to the animals (1).

**Evaluation of protection against M. tuberculosis H37Rv and a high-virulence 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 (8,000 cells; data not shown) subcutaneously at the base of the tail. At 60 days postvaccination, the first group of 10 mice was challenged through the intratracheal route with 2.5 × 104 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 intratracheally infected with the same dose of either H37Rv or the Beijing strain. Three mice per group were euthanized, and their lung homogenates were used to determine the infecting dose which resulted in around 2.1 × 105 to 2.3 CFU (not shown). After 2 and 4 months postchallenge, 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 the statistical significance of CFU, histopathology, and cytokine expression; a P value of <0.05 was considered significant.

**RESULTS**

**Characterization of sigE mutant pathogenicity after intratracheal administration.** In order to characterize the attenuated nature of the sigE mutant in our model, groups of BALB/c mice (70 per group) were infected intratracheally with 2.5 × 105 CFU of H37Rv, the sigE mutant, or its complemented strain. All the animals infected with the sigE mutant survived after 4 months of infection. In contrast, mice inoculated with the complemented or parental strain started to die at 3 weeks postinfection, and all had died by 8 weeks (Fig. 1A). These survival rates correlated well with the CFU in lung homogenates. During the first and second weeks of infection, similar numbers of CFU were detected in the three groups, whereas after days 21 and 28 postinfection, significantly lower bacterial loads were found for mice infected with the sigE mutant than were detected for animals infected with the parental or complemented strain (Fig. 1B). At days 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 infiltrates predominantly constituted by lymphocytes and activated macrophages (large cells with a compact cytoplasm and nucleus with apparent nucleoli) in the alveolar-capillary interstitium and around small blood vessels and bronchial walls after 1 and 2 weeks of the infection with either of the strains, with levels 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 strain showed granulomas after 2 weeks of infection. After 28 days postinfection, only H37Rv induced a significant pneumonia, involving about 30% of the lung surface. In contrast, in mice infected with the sigE mutant, well-formed granulomas and comparable pneumonia appeared only after 60 and 120 days postinfection, when the animals infected with H37Rv or the complemented strain were all dead (data not shown).

**Evaluation of cytokine, iNOS, and β-defensin expression during infection.** The amounts 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 those of animals infected with the parental or complemented strain, they showed a significantly 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 mutant-infected mice but only from 21 and 28 days after the infection (Fig. 2). The only cytokine shown to be expressed at a significantly lower level in the sigE mutant-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 8,000 CFU of either of these two bacterial strains. Two weeks after inoculation, animals which received the \( \text{sigE} \) mutant showed a significant, 2-fold-higher bacterial load at the inoculation site and in the lungs. However, at days 30 and 60 postvaccination, the two groups of vaccinated animals showed similar bacillary loads in the inoculation site, inguinal lymph nodes, spleen, and lungs (Fig. 3), suggesting that the \( \text{sigE} \) mutant is not more virulent than BCG in mice. To further investigate the virulence potential of the \( \text{sigE} \) mutant strain compared to that of BCG, we compared the survival rates of nude mice (20 per group) inoculated subcutaneously with 8,000 CFU of either of the two bacterial strains. Results, shown in Fig. 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 in survival between the two groups, suggesting that the \( \text{sigE} \) mutant is more attenuated than BCG in these immunodeficient animals.

**Comparison of \( \text{sigE} \) mutant and BCG immunogenicity following vaccination.** In order to compare the efficiency of cellular immunity activation induced by the \( \text{sigE} \) mutant or BCG vaccination, we quantified by ELISA the IFN-\( \gamma \) production in cell suspensions collected from the 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 \( \text{sigE} \) mutant stimulated with culture filtrate antigens (CFA) or with the other recombinant antigens produced significantly higher levels of IFN-\( \gamma \) than those of BCG-vaccinated mice at day 60 postvaccination. Since BCG lacks the ESAT-6 structural gene, animals vaccinated with this strain did not produce or secrete any significant amount of IFN-\( \gamma \) after stimulation with this antigen.

**Comparative protection against \( M. \text{tuberculosis} \) H37Rv or Beijing 9501000 in BALB/c mice vaccinated with the \( \text{sigE} \) mutant or BCG.** In order to compare the levels of protection induced by BCG and the \( \text{sigE} \) mutant, groups of BALB/c mice (40 per group for 2 separate experiments) were vaccinated subcutaneously in the base of the tail with 8,000 CFU of either strain. At 60 days postvaccination, mice were challenged intratracheally with 2.5 \( \times \) 10^5 CFU of \( M. \text{tuberculosis} \) H37Rv. Ten mice were euthanized at 60 or 120 days postchallenge. 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 at both time points.

After 4 months postchallenge, 98% of the mice vaccinated with the \( \text{sigE} \) mutant were still alive, while 20% of BCG-
vaccinated mice had died. All nonvaccinated controls died after 11 weeks of infection (Fig. 6A, left panel). These results correlated well with lung bacillary loads and histopathology, showing significantly fewer CFU and less pneumonia for mice vaccinated with the \( \text{sigE} \) mutant than for BCG-vaccinated or control nonvaccinated animals. In particular, \( \text{sigE} \) mutant-vaccinated mice showed a reduction in lung CFU of 1.1 and 1.0 log\(_{10}\) with respect to BCG-vaccinated mice at 60 and 120 days postinfection and a reduction of 1.4 log\(_{10}\) with respect with nonvaccinated controls at day 60 postinfection (Fig. 6B and C, left panel).

In a second vaccination experiment, animals vaccinated following the same protocol were challenged with the highly virulent \( \text{M. tuberculosis} \) strain Beijing 9501000. Nonvaccinated animals started to die after 4 weeks from the challenge, and after 6 weeks all were dead. Mice vaccinated with BCG showed a 30% survival rate after 4 months postchallenge, whereas animals vaccinated with the \( \text{sigE} \) mutant exhibited a significantly higher survival rate of 80% (Fig. 6A, right panel). These results were in agreement with lung CFU determinations (Fig. 6B, right panel): after 60 days from infection, mice vaccinated with the \( \text{sigE} \) 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, \( \text{sigE} \) mutant-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, such as IFN-\( \gamma \) and TNF-\( \alpha \), in \( \text{sigE} \) 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, such as PhoP-PhoQ (38), and \( \sigma \) factors (28).

\( \sigma^{\text{II}} \), 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, including activities such as mycolic acid biosynthesis, fatty acid degradation, membrane protein quality control, and membrane stabilization. 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 potential of this mutant as an attenuated vaccine. Our BALB/c mouse model of progressive pulmonary tuberculosis is suitable for determining 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 correlates well 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 sigE mutant is highly attenuated, permitting complete survival of the infected animals after 4 months of infection, with significantly lower bacillary loads and less tissue damage than for animals infected with the parental and complemented strains. Despite the observation that lungs of mice infected with the sigE mutant had a lower bacterial burden and inflammation, they exhibited significantly higher expression of IFN-γ and TNF-α than the lungs of mice infected with the parental or complemented strain, suggesting that the sigE mutant elicits a stronger immune response. Moreover, the fact that increased levels of IFN-γ and TNF-α were already detectable after 1 day of infection underscores the ability of the sigE mutant to very rapidly induce 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, sigE mutant-infected cells exhibited higher expression of the transcriptional factor T-bet and, in consequence, more IFN-γ production. Moreover, IFN-γ-activated macrophages infected in vitro with the mutant strain induced high expression of TNF-α (13), which could explain the reason for the high induction of iNOS expression that we detected in the sigE mutant-infected lungs. Interestingly, during the late stage of infection, the lungs of mice infected with the sigE mutant showed higher expression of IL-10, an anti-inflammatory cytokine that may limit migration of lymphocytes and reduce tissue damage but that under certain conditions can also exert stimulatory effects on CD4 and CD8 T cells, leading to increased IFN-γ 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 sigE mutant (14).

Another interesting observation was the increased expression of β-defensins 3 and 4 in the lungs of mice infected with the 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 \textit{in vitro} with the \( \text{sigE} \) mutant upregulate genes encoding Toll-like receptors 1 and 2 and \( \beta \)-defensins (13). Finally, we showed that mice infected with the \( \text{sigE} \) 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 Th1 response plus the high expression of \( \beta \)-defensins in mice infected with the \( \text{sigE} \) mutant could be the basis of its attenuation, allowing 100% survival in association with very low CFU numbers and tissue damage. It is worth noting that \( \text{sigE} \)-complemented strain-infected animals produced IL-4 at levels higher than those produced by animals infected with H37Rv; this might be explained by preliminary unpublished data suggesting that in the complemented strain, the level of \( \text{sigE} \) expression is higher than that in the wild-type strain.

These observations justify the hypothesis that the \( \text{sigE} \) mutant could have 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 Th2 response in the presence of a strong Th1 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 significantly 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 immunocompromised patients, so a new attenuated vaccine strain should be more attenuated than BCG in this kind of patient. 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 sigE mutant were more efficient in the production of IFN-γ than those from animals vaccinated with BCG. Taken together, these observations suggest that the sigE mutant is safer and more immunogenic than BCG.

Beside the downregulation of the genes in the α regulon, some of which are involved in surface biology, σE absence has a pleiotropic effect on the bacterial surface, as demonstrated by the transcriptional profile of the sigE mutant after in vitro macrophage infection, showing the induction of genes related to cell wall structure and protein secretion (13). Thus, the 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 secreteme of the sigE mutant and wild-type parental strain, H37Rv, in order to identify differences that could be the basis of their different pathogenic and immunogenic behaviors.

Several mycobacterial mutants have already been demonstrated to have good potential as new efficient vaccines (reviewed in reference 10), and three of them have been analyzed using the model of infection used in this work: (i) a mutant lacking phoP, which was able to induce protection similar to that of BCG (1); (ii) a mutant lacking fadD26 (lacking the cell wall lipid complex phthiocerol dimycocerosate), which conferred 70% survival after 4 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 4 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% mouse survival after 4 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. Preexposure to these environmental mycobacteria probably resulted in a basal level of immunization against mycobacterial antigens, which has 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 that of 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 strain in this strain could represent valuable strategies for further development.

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