Extended safety studies of the attenuated live tuberculosis vaccine SO2 based on phoP mutant

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\textbf{A B S T R A C T}

Safety is one of the main concerns for attenuated live vaccine candidates. Here we extend the stability and attenuation studies of the promising tuberculosis vaccine candidate based on Mycobacterium tuberculosis \textit{phoP} mutant strain, SO2. Stability of the \textit{phoP} mutation was tested after sub-culturing SO2 strain for 6 months in laboratory media and also after 3 months of infection in SCID mice. Results showed no reversion of the \textit{phoP} mutation either in vitro or in vivo. In addition, SO2 was fully sensitive to four major first-line antituberculous drugs against tuberculosis. Safety and toxicity studies were performed in guinea pigs. Animals were infected with a quantity of SO2 equivalent to 50 vaccination doses (2.5 × 10\textsuperscript{6} CFUs) and weight was monitored for 6 months. All animals survived and no histological lesions were found, showing full attenuation of SO2. Studies in a post-exposure model of guinea pigs and mice, previously infected with \textit{M. tuberculosis}, were performed and no toxicity effects were found after inoculation of SO2. All these results together confirm that SO2 has a secure safety profile that encourages its use in clinical trials.

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\section{1. Introduction}

Since the beginning of the XXI century Tuberculosis (TB), AIDS, and Malaria have been considered the three poverty related infectious diseases which cause major Public Health problems. TB disease is caused by \textit{Mycobacterium tuberculosis}. Differently to AIDS and Malaria, there is a vaccine in use today for the prevention of TB in humans. The present vaccine, BCG (Bacillus Calmette-Guérin), is an attenuated live vaccine derived from a clinical isolate of the causative agent of TB in cows: \textit{Mycobacterium bovis}.

BCG has been the vaccine most used in history [1]. So far more than one billion doses of BCG have been already administered, probably as a consequence of the exemplar non-profit distribution started from the very beginning that lead to its immediate production in different laboratories all over the world [2]. BCG is saving thousands of lives each year but its benefit seems to be linked to the prevention of severe disease in children such as meningitis and disseminated TB, with its use being recommended by WHO in countries with high incidence of TB [3]. Unfortunately, protective efficacy generated by BCG in adults against pulmonary forms of TB, the main transmissible form of the disease, is variable [4,5]. One of the reasons for this lack of efficacy is related to the gene deletion suffered by the BCG in its process of attenuation that lead, for instance, to the loss of more than one hundred genes, which include the RD1 region that codifies for a strong immunogenic antigen complex, ESAT-6 [6,7]. In fact, when ESAT-6 was introduced in recombinant BCG, the levels of protection surpassed the conferred by BCG in the guinea pig model [8].

The utility of live vaccines to induce a strong and long-lived immunity against intracellular pathogens has been widely proven [9,10]. Due to the extensive experience of more than 80 years of production, distribution and use of BCG, live vaccines – conferring better protection against pulmonary forms of tuberculosis – offer an enormous potential to replace BCG.

The attenuation of \textit{M. tuberculosis} in a manner that such major antigens would still be kept, is a logical solution for the improvement of the BCG vaccine. Inactivation of \textit{phoP} gene, coding for a virulent transcription factor in \textit{M. tuberculosis}, has already been proven [11]. Its efficacy has been tested in the prophylaxis of \textit{M. tuberculosis} infection in experimental models of mice and guinea pigs.

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The two-component PhoP/R system plays an essential role in M. tuberculosis virulence [12]. Indeed, recent observations have demonstrated that a point mutation in phoP may explain the avirulence of the H37Ra strain [13–15], which is worldwide used as an attenuated counterpart of the reference strain H37Rv. PhoP controls the synthesis of complex mycobacterial lipids implicated in the virulence of M. tuberculosis, which exhibit a potential immunomodulatory activity in cell-mediated immunity [16]. Additionally, a recent work has demonstrated that PhoP is a global regulator of key functions required for the successful intracellular survival of M. tuberculosis within host cells [17].

Preclinical studies conducted in 2001–2006, in different animal models have demonstrated the proof-of-principle that live vaccines based on the inactivation of phoP are highly attenuated and vaccination with SO2 protects against challenge with M. tuberculosis [18]. We have previously studied the attenuation of the phoP mutant in severe combined immunodeficiency (SCID) mice by intravenous and aerosol route of infection [11]. Remarkably, the SO2 mutant showed much lower virulence compared with BCG Pasteur in SCID mice. These studies also showed that complementation of the mutant with the wild-type phoP gene restored the virulence of this strain, confirming that the attenuated phenotype was due to the phoP mutation. Taken together, these studies suggest that safe, attenuated live vaccines based on phoP inactivation show an enormous potential as new vaccine candidates against TB, as so far have demonstrated higher protection activity when compared with BCG [11].

The objective of the present study is to provide further evidence for the safety of SO2, while demonstrating its stability through time, lack of toxicity in the standard toxicity test in guinea pigs used for BCG production, and in mice or guinea pig models of post-exposure infection.

2. Methods

2.1. Primers used in this study

The pair of primers PhoPF 5’aacctgatacgcatacccc3’ (−1000) and PhoPR 5’aacctgaccgaaatgaa3’ (+1046) was used for PCR amplification of the phoP gene. The position of primers with respect to the phoP translational start site is given in parenthesis.

2.2. Drug sensitivity test

The drug susceptibility test was carried out as described in [19]. Briefly, the inoculum was prepared from a fresh 7H9 broth culture, adjusted to a McFarland tube No.1 and diluted 1:20; 10 μl was used as the inoculum. One hundred microlitres volumes of 7H9 broth were dispensed in each well of a sterile 96-well flat bottom plate and serial two-fold dilutions of each drug were prepared directly on the plate by adding 100 μl of the working solution of each drug to achieve the final concentrations. The range of concentrations tested was: 0.008–2.0 μg/ml for rifampicin, 0.008–2 μg/ml for isoniazid, 0.062–32 μg/ml for ethambutol, and 0.016–8 μg/ml for streptomycin. Then, 100 μl of the inoculum was added to each well. A growth control containing no antibiotic and a sterile control for the effect. The two-component PhoP/R system plays an essential role in M. tuberculosis virulence [12]. Indeed, recent observations have demonstrated that a point mutation in phoP may explain the avirulence of the H37Ra strain [13–15], which is worldwide used as an attenuated counterpart of the reference strain H37Rv. PhoP controls the synthesis of complex mycobacterial lipids implicated in the virulence of M. tuberculosis, which exhibit a potential immunomodulatory activity in cell-mediated immunity [16]. Additionally, a recent work has demonstrated that PhoP is a global regulator of key functions required for the successful intracellular survival of M. tuberculosis within host cells [17].

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2.3. Safety in guinea pigs

200–240 g spf Dunkin-Hartley guinea pigs (Harlan Iberica, Sant Feliu de Codines, Catalonia, Spain) were inoculated intramuscularly with 50 times than usual vaccination dose of SO2 (as recommended by European pharmacopeia on the texts of virulence mycobacteria for BCG vaccine freeze-dried production). Animals were housed under spf conditions and provided with sterile water and food ad libitum until the end of the experiment (6 months). Animals were weighed every week to assess the toxicity of our vaccine candidate, taking into account that a 20% weight loss is a symbol of toxicity effect.

2.4. Measurements of delayed hypersensitivity response (DTH)

Guinea pigs were shaved on the back and given intradermal injections of 0.1 ml of PPD (0.5 μg, batch RT-49). The reactions were read after 24 h by measuring the larger transverse diameter of the induration. Mice received an injection of PPD (batch RT-49, 5 μg) into the hind footpad. Swelling at the hind footpad was measured by an engineer’s micrometer before and 24 h after infection.

2.5. Lack of toxicity in a post-exposure model of guinea pigs

200–240 g spf Dunkin-Hartley guinea pigs (Harlan Iberica, Sant Feliu de Codines, Catalonia, Spain) were infected with an M. tuberculosis H37Rv Sanger strain through aerosol inoculation, induced by a Middlebrook device, which inoculated approximately 10 bacilli in the lungs. All animals were treated with isoniazid and rifampicin (10 and 25 mg/kg, respectively) from week 4 until week 8 and then 2 groups of 8 animals were defined. The control group was only treated with chemotherapy, and the vaccinated one was inoculated with SO2 1 × 10^6 CFUs via subcutaneous route at the end of the antibiotic treatment (week 8). Animals were weighed weekly to check their health. They were sacrificed at week 24 after an intraperitoneal injection of ketamine/diazepam (3/1) overdose and we took lung, hilar lymph node and spleen samples to quantify the CFUs and determine the pathology scoring [20].

2.6. Lack of toxicity in a post-exposure model of mice

Six week-old female spc C57BL/6 mice (Harlan Iberica, Sant Feliu de Codines, Catalonia, Spain) were infected with a M. tuberculosis H37Rv Sanger strain through a low dose of aerosol inoculation, induced by a Middlebrook device. All the animals were treated with isoniazid and rifampicin (10 and 25 mg/kg, respectively) once a week from week 6 until week 14 and then 2 groups of 12 animals were defined. The control group was inoculated with sham and the vaccinated group was inoculated with 1 × 10^3 CFUs of SO2 via subcutaneous route at the end of the antibiotic treatment (week 14). Twelve mice per group and time point were sacrificed after being euthanized with halotane (Zeneica Farma, Pontevreda, Spain) overdose at weeks 6, 14, 16 and 19. Lungs and spleens were extracted to quantify the CFUs and to obtain samples for histology.

2.7. Colony forming units (CFUs)

To quantify the CFUs, the sample homogenates were diluted in sterile water and plated on nutrient Middlebrook 7H10 and 7H11 agar (Biomedics s.l., Madrid, Spain). When required, Kanamycin (Km 20 μg/ml), was added to the plates. Bacterial colony forming units were counted after 21 days of incubation at 37 °C.
2.8. Histometry in mice

Samples were fixed in buffered formalin and embedded in paraffin for histometry, 5 μm thick sections from each specimen were stained with hematoxylin–eosin and photographed at 6× using a Stereoscopic Zoom SMZ800 microscope (Nikon, Tokyo, Japan) and a Coolpix 990 digital camera (Nikon). Sections of 8 lung lobes were studied in each case. A sequence of appropriate software programs was used: Scion Image (Scion Corporation, Frederick, Maryland, USA) and Photoshop 5.0 (Adobe Systems Incorporated, San José, California, USA), to determine the area of each single lesion and the total tissue area on photomicrographs. Sections were blindly evaluated in order to get a more objective measurement.

2.9. Animal health

Mice were weighed once a week. They were supervised every day under a protocol paying attention to weight loss, apparent good health (bristled hair and wounded skin) and behaviour (signs of aggressiveness or isolation). Animals were euthanized with halothane (Fluothane, Zeneca Farma) overdose so as to avoid any suffering. Sentinel animals were used to check SPF conditions in the facility. Tests for 25 known mouse pathogens were all negative. All experimental proceedings were approved and supervised by the Animal Care Committee of “Germans Trias i Pujol” University Hospital in agreement with the European Union Laws for protection of experimental animals.

2.10. Statistical analysis

SigmaStat 3.5 (Systat Software Inc., San José, CA, USA) was used to compare values. Statistical significance was determined using paired two-tailed Student’s t-test or One Way Anova test. Differences were significant when marked with * for p < 0.05.

3. Results

3.1. The phoP mutation in SO2 is genetically stable

The SO2 strain (MT103phoP::km) was constructed by inserting a Kanamycin resistance (Km2) marker into the Bcl site of the phoP gene [12]. Since this knock-out strain consist of a single Kmr insertion, the loss of this marker may result in the regeneration of the phoP gene and consequently in the reversion to a virulent phenotype. In order to study genetic stability of the phoP mutation, SO2 was grown in 7H9-ADC-0.05% Tween 80 liquid media without antibiotic (Km). Sub-cultures were propagated every 4 weeks for 6 months and plated onto 7H10-OADC. After replica plating of the colonies onto Km 7H10 plates, all colonies tested were Km resistant. Additionally, DNA from every sub-culture was extracted and PCR-amplified with PhoPF and PhoPR primers (see sequence above). A 3.4 Kb fragment was obtained in all cases, indicating the presence of the antibiotic marker inserted in the phoP gene (Fig. 1).

Once known the in vitro stability of the phoP mutation, we sought to investigate this observation in an in vivo model of infection. Previously we have demonstrated that a dose of 5.4 × 106 CFUs of SO2 inoculated via a lateral tail vein does not cause mortality in the severe combined immunodeficiency (SCID) mice after 120 days of observation [11]. In the same experiment SCID mice were sacrificed after 3 months and spleen and lungs were plated onto 7H10 plates without antibiotic. Colonies grown in this media were subsequently replicated onto plates containing Km, and a hundred percent of the colonies were Km resistant indicating no loss of Km cassette after SCID passage for three months.

3.2. SO2 is fully sensitive to first-line antituberculous drugs

In order to ensure therapy in case of vaccine-associated disease, we decided to test the sensitivity of the SO2 mutant to four major first-line drugs against TB. The sensitivity levels of H37Rv, SO2, and MT103 M. tuberculosis strains against ethambutol, isoniazid, rifampicin and streptomycin were tested using microtiter assay and resazurin as an indicator of cell growth [19]. SO2 was found to be fully sensitive to all major antituberculous drugs. No differences were found in the minimum inhibitory concentration (MIC) for ethambutol, rifampicin and streptomycin in SO2 with respect to wild type MT103. Indeed, SO2 was more sensitive to isoniazid than M. tuberculosis MT103 wild-type and H37Rv (Table 1).

3.3. Inoculation of SO2 does not cause toxicity in the long-term model of safety in guinea pigs

The guinea pig model of infection is extremely susceptible to M. tuberculosis and dies from only one or two viable bacteria; accordingly, this model is used to test the attenuation degree of vaccine candidates [10]. Six guinea pigs were inoculated with 2.5 × 106 CFUs of SO2 (50 times the usual vaccination dose) and weight of the inoculated animals was followed up for six-month. Results revealed no
toxicity as no decrease in this value was detected (Fig. 2). No macroscopic or microscopic lesions were observed at the inoculation site. In addition, once the animals were sacrificed at the end of the experiment, we neither observed macroscopic and microscopic lesions in lungs, livers and spleens. To confirm proper immunity in inoculated animals, DTH response was measured and showed reactivity in all animals 24 h before the end of the experiment (13 ± 2 mm).

3.4. Inoculation of SO2 in a post-exposure infection model of guinea pig does not induce toxicity

Early studies by Robert Koch with *M. tuberculosis* using guinea pigs demonstrated that an intradermal challenge with the tubercle bacillus at 4–6 weeks after the initial infection resulted in local necrosis and in the original tuberculosis lesion. This observation was named the “Koch phenomenon” and also occurs in humans. Indeed, it has been reported that vaccination with BCG aggravates pre-existing disease [21]. Here we conducted experiments in guinea pigs previously infected with *M. tuberculosis* to rule out the possibility of a “Koch phenomenon” against SO2. The inoculation of SO2 did not produce any toxicity in the guinea-pig model of latency. Results are based on the control of bacillary load after the inoculation of SO2 in guinea pigs previously infected with H37Rv and subsequently treated with isoniazid and rifampicin for 4 weeks. Comparing the control (unvaccinated) and SO2 experimental groups (*n* = 8), only one animal was lost in each group and the weight curves did not experience any decrease (Fig. 3). No differences could also be seen between both groups regarding the CFUs counts in the lungs, spleens and hilar lymph nodes at the end of the experiment (week 24 post-infection) (Fig. 4). Remarkably, the lung pathology scoring [20] was lower in the SO2 group (119 vs. 175 units), thus reflecting that inoculation of SO2 in this post-exposure model did not induce more toxicity than the control.

3.5. SO2 does not cause toxicity when inoculated in a post-exposure infection model of mice

Inoculation of SO2 in mice previously exposed to *M. tuberculosis* also did not induce toxicity. Five weeks after the SO2 inoculation (week 19), no difference in the CFUs counts was observed between unvaccinated and SO2-vaccinated groups (Fig. 5). Differently to the unvaccinated controls, inoculation of SO2 was able to induce a DTH...
significant differences could be detected between both groups.

area of the lobes multiplied by 100. Values represent the mean value and standard age was obtained after dividing the area of granulomatous infiltration by the total infiltration were seen between both groups. The pulmonary infiltration percent-

H37Rv but not vaccinated with SO2. No differences in the percentage of pulmonary

in the lung of aerosol-

Fig. 6. Quantification of the granulomatous infiltration in the lung of aerosol-

Control SO2

Fig. 5. Bacillary concentration in a murine post-infection model. After a short period of chemotherapy, mice were inoculated with SO2, which did not cause any interfer-

cence in the CFUs counts. Control group was infected with H37Rv but not vaccinated with SO2. No colonies were obtained on Km plates indicating CFUs correspond to H37Rv and not SO2. Data were expressed as the mean ± standard deviation (SD). No significant differences could be detected between both groups.

response, indicating that SO2 is able to elicit a proper immune response in the post-exposure model of mice (data not shown). In addition, we show that SO2 inoculation did not induce an increase in the granulomatous infiltration of the lung (Fig. 6). Qualitative evaluation of the histology also showed no difference between both groups (data not shown).

4. Discussion

The impact of a new vaccine with a better protection than BCG against the pulmonary forms of tuberculosis could be enormous on the control of the disease [3]. From prime boost strategy, intending to improve the efficacy of BCG, to a new generation of live vaccines with a better protection than BCG and that could replace BCG at mid term, live vaccines are the corner stone of the research on a new TB vaccine [22]. After the Geneva Consensus in 2004, there is a renewed optimism concerning the use of live vaccine candidates to enter Phase I clinical trials [23].

Advances in the understanding of M. tuberculosis biology have made it possible to rationally attenuate this pathogen. Three major strategies are currently being used: (i) the use of auxotrophic mutants, (ii) inactivation of the secA gene and (iii) the use of M. tuberculosis phoP mutants. Auxotrophic mutants which are infective but display limited replication within the host are good vaccine candidates. A representative of this strategy is the mc²6020 strain constructed by inactivation of the panCD and lysA genes involved in pantothenate and lysine metabolism respectively. Phase 1 clinical trials for this vaccine candidate were scheduled for 2006 [22]. The second strategy to rationally attenuate M. tuberculosis consists on inactivation of secA. This gene is a component of a mycobacterial protein secretion system involved in inhibiting the host immune system and consequently, promoting M. tuberculosis survival within the host. Inactivation of secA results in increased host cell apoptosis and increased priming of CD8+ T cells. The secA mutant is currently in preclinical development [24].

The two-component system PhoP/R was found to play an essential role in M. tuberculosis virulence since phoP inacti-

viation conferred an extremely attenuated phenotype in mice macrophages [12]. Initially phoP was studied because a M. bovis strain, which caused XDR-TB outbreaks, demonstrated an increased expression of the phoP gene [25–27]. Recent work on the role of PhoP have demonstrated that this transcription factor regulates a variety of functions in M. tuberculosis including the synthesis of virulence-associated lipids [14,16], the secretion of the major T cell antigen ESAT-6 [13] and a complex virulence network required for intracellular survival of this pathogen [17].

Our previous results showed that the M. tuberculosis phoP mutant, SO2 strain, conferred, with a single dose, better protection than BCG against M. tuberculosis infection in the high dose aerosol guinea-pig model [18]. This protective efficacy was measured by different parameters, such as extended survival at the end point of the experiment, decreased pathology in lungs after M. tuber-

lossis challenge and decreased CFUs of M. tuberculosis in the lungs of SO2-vaccinated animals [11]. Additionally, SO2 has also shown a decreased toxic activity when compared with BCG upon examining the survival in an intravenous challenge model of SCID mice [11]. Moreover, the inability of SO2 to infect immunocompromised SCID mice by aerosol route [11] is indicative of the loss of capacity of this strain to propagate into the environment and the population.

Safety is one of the main concerns and a major challenge for attenuated live vaccines. In this work, we extend the safety studies of the SO2 strain in order to provide enough assurance for its future use in humans. Since rearrangements in the phoP mutation could happen in a live vaccine after sub-cultivation, we decided to study the stability of this mutation after successive passages in culture media. Our results indicated no loss of the Km and cassette in the 6 months of the study. Additional in vivo studies showed that 3 months after intravenous inoculation of SO2 in immunocompro-

mised SCID mice, the Km resistant phenotype was conserved. These data indicate that Km insertion within the phoP gene was genetically stable during time in vitro and in vivo. However, even though chromosomal mutations are genetically stable in M. tuberculosis, the potential release of antibiotic resistance markers from genetically modified organisms into the environment and the subsequent transfer to other organisms is an important concern. The Geneva Consensus recommends non-reverting and unmarked mutations for live attenuated vaccines based on M. tuberculosis [23]. In this context, we are currently working on an isogenic vaccine candi-
date which carries a deletion in the \textit{phoP} gene without antibiotic resistance marker.

Another interesting question, which arises, is whether after genetic modification of \textit{M. tuberculosis} strains – and particularly the SO2 mutant – the sensitivity profile to antibiotics could be changed. The SO2 strain was found to be fully sensitive to ethambutol, isoniazid, rifampicin and streptomycin. SO2 was more sensitive to isoniazid than \textit{M. tuberculosis} wild type and this could be due to changes in the cell envelope of the \textit{phoP} mutant [12,16,28]. All these results indicate that SO2 strain is sensitive to major antituberculous drugs and in case of potential infection with SO2-based vaccines it would be possible to be treated.

Inoculation of a high dose of vaccine candidates in guinea pigs is a study inspired by the BCG validation standards for toxicity. In this study, animals were inoculated with 2.5 × 10^6 CFUs of SO2 (50 × the standard vaccination dose). Our data indicate the lack of toxicity of SO2 strain since the health status of the animals was satisfactory, as evidenced by the constant increase of their weight and the lack of pathology after the end of a 6 months follow up. Determination of DTH in guinea pigs at the end of this study reflected similar values to the ones obtained 4 [29] or 6 weeks [30] after immunization with BCG, thus reflecting the conservation of the immune response. Even aerosol infection of guinea pigs with a low dose of \textit{M. tuberculosis} in our own lab has triggered an equal DTH response (data not shown).

The use of post-exposure infection models for checking toxicity when vaccines are administered in a therapeutic way is based on previous data showing that this administration can be dangerous because of potential induction of the “Koch phenomenon” [31,32]. A number of vaccine candidates have recently been tested to assess the effectiveness and lack of toxicity after post-exposure vaccination [33]. The results suggest that although most vaccine candidates are unlikely to evoke the “Koch phenomenon”, extreme caution should be taken to avoid serious reactions in previously infected individuals in clinical trials. We have used previous validated models of post-exposure infection in guinea pigs and mice [34,35] to address this question, and we can conclude that no toxic effects have been developed in any of the cases, as it has been demonstrated after examining the bacillary concentration and histology of the tissues. In fact, in the guinea pig post-exposure model, the administration of SO2 decreased the pathology, which could be related to a kind of protective effect, although this was not confirmed by a reduction in the bacillary counts. In any case, the lack of toxicity in these models gives an idea about how safe this vaccine is, including the potential secure profile to be used in subjects with latent tuberculosis infection.

Preclinical testing of live vaccines based on \textit{phoP} inactivation using SO2 as vaccine has demonstrated proof-of-concept, with a high degree of attenuation and protection against disease in various animal models [36]. Extended safety studies presented here encourage the use of SO2 strain as a starting point for the construction of a next generation attenuated live vaccines following the Geneva Consensus [23]. In this context, we are currently working on the introduction of a second mutation in the SO2 mutant and the subsequent elimination of the antibiotic markers, with the objective to take these vaccines toward clinical trials.

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References


