Vaccines & Antibodies

Live tuberculosis vaccines based on *phoP* mutants: a step towards clinical trials

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Bacillus Calmette–Guérin (BCG) is the only preventive treatment for tuberculosis in humans, but this live vaccine confers variable protection against pulmonary tuberculosis in adults. Advances in the understanding of *Mycobacterium tuberculosis* immunopathogenesis have renewed hopes of developing new prophylactic vaccines conferring better protection than BCG. The authors describe here state-of-the-art attenuated live vaccines based on inactivation of the *phoP* gene, a transcriptional regulator of key virulence networks in *M. tuberculosis*. Recent preclinical testing of live vaccines based on *phoP* inactivation has demonstrated proof of concept, with a high degree of attenuation and protection against disease observed in various animal models. These results demonstrate that *phoP* mutants are promising new live vaccines for tuberculosis prevention. The steps that now need to be followed, to take these live vaccines towards clinical trials, are also reviewed, together with the potential of these vaccines to replace BCG.

Keywords: attenuated vaccines, BCG, tuberculosis, two-compartment system, vaccine


1. Introduction

Despite hopes of its eradication, tuberculosis (TB) remains a leading cause of death from infectious diseases worldwide. The World Health Organization (WHO) declared TB an emerging public health problem in 1993, and TB has now reached alarming proportions, with a growing number of cases and deaths linked to HIV in some of the poorest countries in the world [1,2].

The etiological agent of TB, *Mycobacterium tuberculosis* (MTB), is a facultative intracellular pathogen that has successfully adapted to survival in human hosts. One third of the human population is infected with MTB. However, despite this high rate of infection, only ~5% to 10% of infected individuals go on to develop clinical disease, which proves fatal in 30% of patients, resulting in about two million deaths annually [3].

There are three major strategies for dealing with the global burden of TB: drug treatment, chemoprophylaxis and vaccination [4]. Drug treatment aims to cure the patient, ensuring an absence of relapse, to reduce the spread of infection and to prevent the appearance of drug-resistant strains. These goals are achieved through the effective treatment of patients with drug combinations including first-line treatment regimens based on isoniazid and rifampicin. Such treatment regimens have been reasonably successful in the developed world, but it remains very difficult to contain the transmission of infection in many developing countries. This may be partly due to the long-term nature of the treatment, which must be maintained for up to 9 months. In this context, the directly observed treatment short
course (DOTS) approach adopted by the WHO has proved effective for reducing the prevalence of the disease in targeted regions [1]. Although DOTS has slowed the increase in prevalence, it has actually failed to reduce prevalence in Eastern Europe and Africa, mostly due to difficulties in reaching or maintaining the needed standard of care, which makes the development of an effective TB vaccine an urgent priority.

During the 1990s, epidemic cases of multidrug-resistant (MDR) TB, defined as resistance to at least isoniazid and rifampicin, were described in the US and Europe [5,6]. Since then the number of cases of MDR TB has continued to increase worldwide [7,8]. The treatment of MDR TB requires the use of second-line drugs that are less effective, more toxic, and more expensive than isoniazid and rifampicin. Moreover, cases of extensively drug-resistant (XDR) TB, defined as MDR strains also resistant to second-line drugs, have recently emerged and pose a worldwide threat to public health and TB control, raising concerns about a possible future epidemic of virtually untreatable TB [9].

The search for new compounds capable of combating MDR and XDR strains continues, but no new drugs other than rifabutin and rifapentine, not available in most of developing countries, have become commercially available in the 40 years following the release of rifampicin [10]. TB vaccines are, therefore, urgently required to control the spread of TB. There are two potential strategies for vaccination against TB: pre-exposure or prophylactic vaccines, which aim to prevent disease, and post-exposure or therapeutic vaccines, which could be used in combination with antituberculosis drugs [11] to eliminate the infection before the disease develops. Therapeutic vaccines correspond to a special group within post-exposure vaccines to be administrated to patients with active TB in conjunction with antituberculosis treatment in order to shorten the length of anti-TB chemotherapy.

We review here prophylactic live vaccines, focusing on those based on inactivation of the *phoP* gene, a key transcriptional regulator of virulence networks in MTB.

2. The present vaccine against tuberculosis

‘Prevention is better than cure’ should be the underlying philosophy of any healthcare policy tackling disease. Pre-exposure, prophylactic vaccines are one of the most useful and cost-effective tools for reducing the morbidity and mortality associated with infectious diseases [12].

*Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) is at present the only vaccine used for TB prevention in humans. BCG is a live vaccine, and was obtained from a pathogenic strain of *M. bovis* after 230 successive passages in the laboratory between 1908 and 1921. During this period, the bacteria gradually lost their ability to cause disease, but retained their ability to stimulate the immune system, thereby protecting various animals against TB. BCG is an inexpensive vaccine that has been administered since the early 1920s, with > 2.5 billion people immunised with BCG since 1948. BCG seems to be most effective against childhood forms of disease, providing more variable protection against pulmonary TB in adults [4,13,14]. The WHO continues to recommended BCG vaccination in countries with a high prevalence and incidence of TB, precisely because of this efficacy in children. BCG vaccination is at present compulsory in at least 64 countries, and is carried out in > 167 countries. Indeed, BCG remains the most widely used vaccine in the world.

Genomic analyses have been carried out to determine the basis of attenuation in BCG. Several regions have been identified as deleted from BCG but present in MTB, resulting in the loss of > 100 genes [15]. This genomic decay probably occurred during long-term subcultures of BCG. In addition, once BCG had emerged and had been shown to be effective at preventing TB, it was distributed to laboratories worldwide, where its subculture continued.

Subculture of the original BCG strain in different laboratories has led to the appearance of different variants of BCG vaccines, with different immunological properties. This led to the WHO recommending the freeze-drying of BCG vaccine stocks and their storage at -80°C [16]. The first genome sequence of a BCG strain has been published and its comparison with the genome sequences of other BCG strains has demonstrated that differences in immunogenicity are related to genomic diversity [17]. Based on these observations, one can establish a genealogy of BCG strains potentially associated with the degree of attenuation or protection (Figure 1). Five major BCG strains are presently used as vaccines: Danish 1331, Japan (Tokyo 172), Moreau Rio De Janeiro, Russia (Moscow) and Pasteur 1173 (Figure 1) [18]. These BCG strains differ both from each other and from their ancestors, so it is prudent to refer to BCG vaccines in the plural [16].

The different genomic contents of different BCG vaccines may contribute to the variability in protective efficacy observed in animal models [19]. Recently, differential production of lipid virulence factors has been described among BCG vaccine strains and implications on BCG safety have been suggested [20]. Three BCG substrains, BCG Japan, BCG Moreau RJ and BCG Glaxo do not produce phthiocerol dimycocerosates and phenolic glycolipids, important factors for MTB virulence. These three BCG substrains are less likely to induce adverse reactions than other BCG strains such as BCG Pasteur, Sweden or Denmark.

Clinical trials are now underway to compare protection conferred and the dissemination of different BCG strains. A programme based on the use of BCG Danish administered intradermally gave similar levels of overall protection against TB in children to a programme based on the use of BCG Tokyo administered percutaneously, but the proportion of cases with disseminated disease
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**Figure 1. The degree of attenuation and protection of BCG vaccines is associated with BCG genealogy.** This diagram illustrates the evolutionary pathway of BCG strains, showing genomic deletions of RD regions and genomic polymorphism at the *phoPR* locus (black boxes). The recently published genome sequence of BCG demonstrated the presence of two independent tandem duplications: DU1 and DU2. DU1 is restricted to BCG Pasteur. Four variants of DU2 exist, based on which BCG strains can be assigned to different groups, depicted as circled regions (group II has been omitted for clarity) [17]. The comparison between BCG genomes has demonstrated that differences in immunogenicity may result from genomic diversity. Group I BCG strains secrete large amounts of MPB70 antigen, have two copies of the insertion sequence IS6110, and produce methoxymycolate. In contrast, BCG strains from groups III and IV, including BCG Pasteur, Tice, Danish and Glaxo, secrete little MPB70, have a single copy of IS6110 and do not produce methoxymycolate. Group I strains also differ from the other strains in having one of the two copies of the IS6110 element located upstream from the *phoPR* TCS. This may have an effect on *phoP* transcription, as *phoP* is more strongly expressed in BCG Japan than in BCG Pasteur. Conversely, the group of BCG vaccines considered to be the most attenuated, BCG Danish, Glaxo and Merieux, all have a 10 base pair deletion in the *phoR* gene that would affect *phoPR* expression [17]. These observations may account for the immunogenicity of group I BCG strains and the highly attenuated phenotypes of vaccines from groups III and IV. Adapted from [17] Copyright © 2007 by the National Academy of Sciences.

*BCG substrains that do not produce PDIMs and PGLs, important *M. tuberculosis* virulence factors [20].

BCG: Bacillus Calmette–Guerin; *M. bovis*: Mycobacterium bovis; *M. tuberculosis*: Mycobacterium tuberculosis; PDIM: Phthiocerol dimycocerosates; PGL: Phenolic glycolipid; RD: Regions of difference.

was lower in the BCG Danish programme [21]. As the strain and route of administration in these clinical trials were different, further studies are needed in order to discriminate between BCG strains by using the same route of administration.

The reasons for the BCG failure have been widely debated, and remain the topic of active research. One of the reasons for the BCG failure was the natural exposure to environmental mycobacteria, which can influence immune response and interact with the effect of BCG vaccination in developing countries. This theory has been supported by the fact that exposure to environmental mycobacteria is prevalent in those countries where BCG confers low protection, and by a number of studies showing that exposure to environmental mycobacteria has an impact on the protection afforded by BCG in animal models [22].
This phenomenon has been proposed as a plausible explanation for the North–South gradient in the effectiveness of BCG [22]. Host-related differences, such as genetic and host immune status, use of different BCG preparations, diverse levels of nutrition, and socio-economic issues should also impact BCG efficacy in different populations. It has been recently demonstrated that cross reaction is due to antigens shared between BCG and environmental mycobacteria, such as Ag85B, but not deleted antigens of BCG, such as ESAT-6 and CFP10 [23]. Rational attenuated vaccines, which include deleted regions of BCG with major antigens not shared with environmental mycobacteria will overcome the problem of the antagonistic effect of BCG to previous environmental mycobacterial immunisation [23].

Existing problems of substrain variability and the low efficacy of BCG in a number of trials evaluating protection against pulmonary disease [24] clearly indicate that this vaccine is inadequate. With the goal of TB eradication in mind, major efforts are being made worldwide to develop new rationally constructed live vaccines. There are two main research strategies. The first is to improve the efficacy of BCG by prime-boost regimens involving priming with a BCG vaccine and boosting with either a subunit vaccine or a non-replicating viral vector-based delivery system. The second strategy involves the development of a new TB vaccine conferring stronger protection against respiratory forms of TB, which could potentially replace BCG in the medium term [25]. The potential impact of these different kinds of vaccines and different strategies has been explored using mathematical modeling. Assuming that vaccines would be available for use in 2015 and efficacy would protect 70% of target population in a country with an annual incidence set at ~200 per 100,000 population in 2015, mass vaccination of uninfected population (pre-exposure) would reduce the annual incidence to 20 per 100,000 in 2050 [4].

3. Research strategies for improving Bacillus Calmette–Guérin

Advances in the fields of immunology and MTB pathogenesis, vaccine studies and technological developments have placed us in a better position for the construction of new effective and safe vaccines against TB [26]. Several groups in a number of countries have embarked on the ambitious project of finding new vaccines providing higher levels of protection than BCG. A large number of vaccine candidates have been proposed as a result of this basic research over the last decade [13,14,25,27].

Two main approaches are being used to improve the TB vaccine: i) subunit vaccines that can deliver immunodominant mycobacterial antigens; ii) live vaccines, either BCG strains genetically manipulated to express immunodominant antigens or MTB attenuated strains created by random mutagenesis or the targeted inactivation of virulence genes.

3.1 Subunit vaccines

Non-viable subunit vaccines (protein or DNA vaccines) have been selected by various rational and experimental approaches. They induce partial protection against experimental TB infection in various animal models [28,29], but most have proved no more effective than BCG. New antigen formulations, including multiple antigens or epitopes, are being investigated and it is hoped that they will afford better protection in humans [30-32].

Although subunit vaccines have proved no more effective than BCG, their use to boost BCG has been suggested and today are promising TB vaccine strategies tested in humans. Prime-boost experiments, using protein subunits in animals previously vaccinated with BCG (BCG+), have given very encouraging results [13,14,33]. This vaccination strategy may have applications for preventing TB reactivation in the elderly.

Enhanced immunogenicity and protective efficacy against MTB have been demonstrated in prime-boost regimens, following boosting with a recombinant modified vaccinia virus Ankara (MVA) expressing MTB antigens. This strategy strongly boosts BCG-induced MTB antigen-specific CD4+ and CD8+ T-cell responses in mice. Protection is correlated with the induction of antigen-specific IFN-γ-secreting T cells in lung lymph nodes [34]. There is a need for developing effective boosting vaccination strategies. Combination of BCG boosted with DNA vectors expressing TB antigens, showed increased specific antimycobacterial immune respose in different animal models [35]. BCG priming was remarkably boosted by intranasal adenoviral-vector expressing Ag85A [36]. A combination of BCG and subunit vaccine consisting of the proteins Ag85B and ESAT-6 in cationic liposomes has been recently tested [37].

3.2 Live vaccines

3.2.1 Live vaccines based on recombinant Bacillus Calmette–Guérin

After a decade of development of genetic tools for manipulating the tubercle bacillus, it is now possible to construct new live vaccines by genetic engineering [38,39]. These vaccines may be recombinant BCG (rBCG) strains genetically manipulated to express immunodominant antigens or attenuated strains of MTB produced by random mutagenesis or the targeted inactivation of virulence genes [40,41].

Various strategies have been used to develop rBCG against mycobacterial diseases [42]. One such strategy is based on rBCG producing large amounts of autologous protective antigens, such as immunodominant MTB antigens. These supplementary antigens are designed to enhance immunity to other BCG antigens by increasing the expression of genes encoding other antigens. The rBCG30 strain, which expresses and secretes Ag85B, a major secreted protein of MTB [42-45],
is associated with better host survival after challenge than parental BCG in the guinea pig model of pulmonary TB. Alternatively, *M. bovis* genes encoding important antigens lost during the production of BCG can be restored. This approach is illustrated by the ESAT-6/CFP10 complex, a secreted T-cell antigen absent from all BCG vaccines. rBCG strains producing and secreting ESAT-6 confer enhanced protection against TB [46]. Both these approaches are attractive for BCG improvement and the addition of new antigens, and may be important in the induction of immunity against TB.

Another interesting approach to the enhancement of BCG immunogenicity is to favour translocation of the bacterium from the phagolysosome to the cytosol, as it has recently been demonstrated that virulent mycobacteria tend to be cytosolic and that a cytosolic distribution may enhance MHC-based antigen presentation [47]. rBCG strains that produce and secrete listeriolysin, a protein from *Listeria monocytogenes* that form pores in biological membranes, are more able to escape from the phagolysosome and display higher levels of antigen presentation, resulting in enhanced T-cell-mediated responses [48,49].

### 3.2.2 Live vaccines based on attenuated *Mycobacterium tuberculosis*

An important consideration in the use of BCG-based vaccines is that five of the six immunodominant antigens of *M. bovis* are either deleted or downregulated in some or all BCG strains (ESAT-6, CFP10, Ag85, MPB64, MPB70, MPB83) [14]. Moreover, regions of difference (RD)1, which is absent from all BCG vaccines but present in MTB, includes ESAT-6 and CFP10, two of the six immunodominant antigens shown to be important for protection against challenge in the guinea pig model [46]. The advantage of using attenuated MTB strains as vaccine candidates is that > 100 genes deleted from BCG are present in MTB [17]. Advances in TB research and the completion of the MTB genome sequence [50] have made it easier to analyse the contribution of individual genes to MTB virulence [51]. The development of genetic tools for inactivating selected genes has made it possible to attenuate MTB strains in a rational manner [38].

Various molecular methods have been developed for the generation of attenuated MTB strains as vaccine candidates [52]. Systematic studies of genes involved in virulence in mice, based on the use of a random signature-tagged transposition library, led to the identification of 16 attenuated mutants [51]. Auxotrophic mutants, requiring exogenous nutrients for survival, are infective but display limited replication within the host; they display various degrees of attenuation and diverse potential as vaccine candidates in animal models [53]. Double auxotrophic mutants have also been described [54-56]. It has also been reported that the secA gene, encoding a component of a mycobacterial protein secretion system, is also involved in inhibiting the host immune response and promoting MTB survival within the host. Conversely, secA gene inactivation enhances host cell apoptosis and increases the priming of antigen-specific CD8+ T cells in vivo. These results pave the way for a new approach to improving live vaccine candidates; the secA mutant is in preclinical development [57].

### 3.2.3 Attenuated live vaccines based on *phoP* inactivation

One of the most difficult questions when designing a strategy for attenuating a clinical isolate of MTB concerns the choice of gene or genes to target from the 4000 or so genes in the MTB genome [50]. The authors focused on a particular *M. bovis* strain (B strain) that had caused unusual MDR TB outbreaks in Europe [6,8]. The B strain was first isolated in 1991 and was responsible for the deaths of > 100 people in an outbreak largely confined to individuals infected with HIV. Unlike other strains of *M. bovis*, the B strain is transmitted between humans through respiration, resulting in high rates of re-infection in TB patients [58]. The first classification of strain B in 1997 defined this strain as an MDR strain, but this strain today fits the definition of an XDR strain [59], having characteristics identical to those of the strain responsible for XDR TB outbreaks in South Africa [60]. These features led the authors to carry out molecular studies of the B strain. The authors observed a remarkable increase in *phoP* gene expression [61], suggesting a role for this gene in MTB virulence.

The *phoP* gene corresponds to Rv0757 in the MTB genome [50] and has been reported to encode the transcription factor of the PhoPR two-component system (TCS). TCS enable the bacteria to detect environmental stimuli and to respond to them, resulting in adaptation. They consist of a sensor protein, which is usually associated with the membrane, and a cytoplasmic transcription factor responsible for the activation or repression of a subset of genes in response to the signal detected by the cognate sensor protein. The MTB genome encodes only 11 TCS [48,49], far fewer than have been found in many other bacteria. The small number of TCS present is almost certainly the result of MTB adaptation to an intracellular lifestyle. Autoregulation has been described for six of the TCS of MTB including *phoPR* [63]. TCS have been reported to play an important role in virulence regulation in other intracellular pathogens [64]. Given this finding and the high levels of *phoP* gene transcription in the B strain, the authors investigated the role of the PhoPR system in MTB virulence.

The authors investigated the association between the PhoPR system and virulence in the MT103 strain, a fully virulent MTB clinical isolate. They inserted a kanamycin cassette into the *phoP* gene to disrupt this gene. The resulting mutant was named SO2 [65]. The authors chose
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1. Construction of live vaccine candidates
2. Safety toxicity
3. Immunogenicity protection

Macrophages (64) → Mice [29,64,71] → SCID mice [71] → Guinea pigs () → Mice [70,71] → Guinea pigs [29,71] → Primate ()

Preclinical testing 2001 → 2007

Figure 2. Steps followed in preclinical testing of the prototype vaccine based on phoP inactivation (SO2). This diagram shows the order of preclinical trials carried out with cellular and animal models to demonstrate rationally the efficacy of live vaccine candidates. Once constructed, live vaccine candidates are tested for attenuation in cell cultures and animal models, including immunocompetent mice, immunocompromised mice and guinea pigs. Sufficiently attenuated candidates are then tested for efficacy in protecting against TB infection in immunocompetent mice, guinea pigs and primates. Preclinical testing of the SO2 vaccine candidate between 2001 and 2007 demonstrated that it had a highly attenuated phenotype [29,65,72] and a protective immunity greater than that conferred by the current BCG vaccine [29,71,72].

to use MT103 rather than the reference strain H37Rv as the parental strain, to avoid the need for subculture in the laboratory, which might lead to strain variability [50].

This mutant displayed marked changes in bacterial and colony morphology, together with impaired multiplication in mouse macrophages in vitro. SO2 was also found to be attenuated in vivo, in a mouse infection model, in which it persisted in the organs but was unable to replicate (Figure 2) [65].

Having demonstrated the essential role of PhoPR in MTB virulence [65], the authors need to consider the molecular mechanisms underlying attenuation. A series of phoP and phoPR mutants were constructed in MTB strains of three genetic backgrounds: H37Rv, MT103 and a Beijing clinical isolate. The phoP mutants displayed major modifications to the lipid composition of the MTB cell envelope, as expected given that PhoP positively regulates the synthesis of complex lipids, such as sulfolipids (SL), diacyltrehaloses (DAT) and polyacyltrehaloses (PAT) [66]. These complex lipids are important virulence factors with immunomodulatory effects on human CD4+ and CD8+ T cells [67-69], interfering with the immune system of the host. These phenotypic results have been confirmed by microarray studies, demonstrating that PhoP positively regulates genes encoding proteins involved in the biosynthesis of SL, DAT and PAT [70] as well as other important virulence factors previously described as lipF, fbpA and mmpL8 [51], confirming the importance of PhoP-regulated genes in MTB virulence [70].

Considering PhoP as a transcriptional factor able to regulate important virulence genes implicated in MTB virulence [51] and since it is inactivation conducts to a full attenuated phenotype in cellular and animal models, we could consider the role of PhoP as an essential regulator of virulence network in MTB.

4. Preclinical studies of live vaccines based on phoP

The authors used SO2 as a prototype vaccine in preclinical studies conducted from 2001 to 2007 with the aim of demonstrating proof of principle that a live vaccine based on phoP inactivation is highly attenuated in various animal models but has potential as a prophylactic vaccine against challenge with MTB (Figure 2).

The protective efficacy of SO2 was studied in Balb/c mice. The results of mouse vaccination experiments indicated that SO2 had vaccine characteristics similar to those of BCG Pasteur [71,72]. Cellular immunity is considered particularly important for protection against TB [73]. The authors therefore carried out flow cytometry to quantify CD4+ and CD8+ responses in Balb/c mice vaccinated with SO2 and to compare these responses with those induced by BCG. Vaccination with SO2 induced a significantly larger number of CD4+ cells than vaccination with BCG, 14 days after the initial inoculation, and a significantly larger number of CD8+ cells after 45 days. Consequently, IFN-γ levels in splenocytes stimulated with MTB culture filtrate were consistently higher in SO2-vaccinated animals than in BCG-vaccinated animals [72].
T helper (T\textsubscript{H}) cells play a key role in immune responses to MTB infection, because they activate macrophages, killing intracellular MTB, and promote the development of cytotoxic T lymphocytes, which kill infected target cells [74]. The T\textsubscript{H}1 subtype is responsible for cell-mediated immunity and T\textsubscript{H}1 cells produce IFN\gamma and IL-2 as the major cytokines protecting against TB [74]. The severity of TB disease is strongly correlated with IL-4 levels, this cytokine working with TNF-\alpha to induce strong inflammatory activity, resulting in the exacerbation of tissue damage [75]. In studies of the immunological responses of Balb/c mice infected with MT103 or SO2, SO2 was found to induce lower levels of IL-4 in the lungs of infected mice, associated with a lower percentage of lungs affected by pneumonia. Levels of IFN\gamma, IL-4 and TNF-\alpha were higher in the lungs of MT103-infected mice, which displayed progressive disease and greater inflammation than SO2-infected animals [71]. These immunological responses were accompanied by a decrease in the bacterial load in the lungs and lower levels of dissemination to other organs in cases of SO2 infection, indicating the potential of SO2 as a protective prophylactic vaccine in mice.

Even if the mouse model provides valuable information about a number of immunological parameters, guinea pigs are widely recognised to constitute a more susceptible model of TB, with many similarities in the progression and pathology of the human disease [76-79]. The two models represent different points on a scale of susceptibility; both models have their advantages but the extreme susceptibility of the guinea pig disease model is thought to make it a very stringent model for vaccine screening [76-79]. Within the European TBVAC (TB Vaccine Cluster) consortium, various vaccine candidates have been compared in the guinea pig model of infection [29]. Animals were immunised by aerosol and challenged with a high dose of MTB. Together with SO2, BCG boosted with two doses of the immunodominant antigen Ag85A was the only one of the 24 TB vaccine candidates tested to confer greater protection than BCG. Survival was significantly longer in guinea pigs vaccinated with a single dose of \textit{phoP} vaccine (SO2) than in animals vaccinated with BCG [29]. Not only did SO2-vaccinated guinea pigs survive longer, but SO2 conferred greater protection than BCG, as shown by the lower bacterial load in the lungs (and spleen) and lower levels of disease, with a lower percentage of lung consolidation and fewer histological lesions. Thus, this vaccine candidate displayed generally good parameters of protection: prolonged survival, lower bacterial load in organs and fewer lesions.

These findings are encouraging, but require confirmation in further studies in non-human primates. SO2 administered as a single dose and prime-boost BCG with MVA expressing Ag85A are being studied in the non-human primate model at the Biomedical Primate Research Centre Rijswijk, The Netherlands (Frank Verreck et al., unpublished results). BCG boosted with Ag85A is the candidate vaccine at the most advanced stage of development [80].

5. Safe live vaccines based on \textit{phoP} mutation

Several major issues, concerning safety and regulatory matters, must be resolved for the use of live organisms as vaccines. This is particularly true for attenuated MTB. The early use of BCG was marked by a tragic accident. In 1927, > 25% of the 250 or so children vaccinated with a particular batch of BCG in Lubeck, Germany developed TB. It was later recognised that this batch had been accidentally contaminated with a virulent strain of MTB [81]. Safety is a key concern in the use of live vaccine candidates. Recent evidence shows that children who were HIV-infected when vaccinated with BCG at birth, and who later developed AIDS, were at increased risk of developing BCG disease [1,81].

Survival studies in immunocompetent Balb/c mice showed the SO2 mutant to be fully attenuated with respect to wild-type MTB [71]. Survival studies in immunocompromised severe combined immunodeficient mice infected with bacterial aerosols showed that animals infected with MTB died within 40 days of infection, whereas animals infected with the \textit{phoP} mutant survived to the end of the experiment (6 months), and no bacteria were recovered from the lungs or spleens of these animals [72]. Additional survival studies in the severe combined immunodeficient mouse model intravenously infected with different doses of SO2 demonstrated this mutant to be even more attenuated than BCG [72]. The potential of attenuated MTB vaccines with increased safety would be of extraordinary importance in order to decrease the risk of vaccine dissemination to HIV-infected population (HIV positive) recipients [82]. The risk of disseminated BCG disease is increased several hundred fold in HIV-infected infants compared with the documented risk in HIV-uninfected infants. Data on the protective effect of BCG in HIV-exposed and infected children is lacking. Population- and hospital-based surveillance is vitally important to more accurately estimate the safety and benefits of BCG in HIV-exposed and infected infants [82].

Extended studies of the safety and toxicity of SO2 have been performed in guinea pigs to demonstrate the lack of toxicity of this mutant. Assays of susceptibility to antituberculous drugs and studies to confirm the stability and lack of reversion of the \textit{phoP} mutation are also underway (unpublished results).

A consensus document was developed at the Geneva conference, to facilitate the transfer of the most promising vaccine candidates from testing to clinical use and the control of TB [18]. A set of criteria were proposed for consideration during the vaccine development process. One of these criteria for live vaccine candidates based on MTB is the presence of at least two non-reverting independent mutations in the mycobacterial genome. Regulatory issues are fundamental for the development of new tuberculosis vaccines [83]. For \textit{phoP}-based vaccines, the authors are working on a new generation of live vaccines.
based on phoP mutation carrying a second additional mutation and the subsequent elimination of antibiotic resistance markers.

6. Expert opinion

TB, AIDS and malaria, the ‘big three’ killer infectious diseases, are considered to be related to poverty. The BCG vaccine is available for TB, but this is not the case for AIDS and malaria. Although TB control programmes are based in early detection of new TB cases and treatment, BCG vaccination programmes are maintained because BCG protects against severe childhood forms of disease, including milliary and extrapulmonary TB and the often fatal TB meningitis, but a new TB vaccine conferring better protection against the pulmonary manifestations of the disease is urgently required to reduce the incidence of the disease in endemic areas.

For the first time since the introduction of BCG vaccination 80 years ago, new TB vaccine candidates are being constructed, tested and evaluated in humans. The development of a new TB vaccine is an integral element of the Global Partnership to Stop TB [84] a network of international organisations, countries, public and private sector donors, governmental and non-governmental organisations and individuals, which aims to develop a safe, effective, licensed vaccine, available at reasonable cost, by 2015. The Stop TB Partnership has recently developed strategic plans for TB vaccine development. A complete list of TB vaccine candidates in the pipeline at TBVAC [85] and Aeras (Aeras Global TB Vaccine Foundation) [86] has been produced by EDCTP (European and Developing Countries Clinical Trials Partnership) [87].

If the goal of having an effective licensed vaccine available by 2015 is to be attained, it is estimated that at least 20 vaccine candidates should enter phase I safety trials, with about half going forward for immunological evaluation in phase II trials and four being evaluated in phase III efficacy trials [4]. Effective BCG prime and boost regimens will be the first candidate assessed. However, there is a need to expand discovery and translational research on TB, to make it possible to design new-generation vaccines to be added to the list of existing vaccine candidates (EDCTP), to ensure constant supply to the pipeline.

The use of live vaccines against TB is likely to benefit from > 80 years of experience in the use of BCG and the high potential of such vaccines to have a beneficial effect. Live vaccines are the cornerstone of all new TB vaccine strategies from prime boost to improve BCG or BCG replacement strategy. Live vaccines are easy to produce at relatively low cost and can easily be distributed to the large populations in which they are most needed in the countries with the highest incidence of TB. All these factors identify live vaccines to be highly valuable potential candidates for combating TB. Previous experience of differences between BCG substrains would require that new vaccines should be monitored for variations in genomic composition.

Safety and regulatory obstacles to the use of live organisms must still be overcome. Following the achievement of a consensus in Geneva, there is renewed optimism that live vaccines could be reliably used against TB. Safe and effective rationally attenuated MTB strains are thus potential candidates to replace BCG in the medium term. HIV-positive recipients are obviously a relevant issue today as HIV/TB co-infection, demands even higher safety demands for a new vaccine, particularly for post-exposure approaches.

Step-by-step preclinical testing of live vaccines based on phoP inactivation has demonstrated a high degree of attenuation and protection against TB in various animal models, indicating that these vaccines are promising for use against TB. A new generation of attenuated live vaccines based on phoP mutation is now ready for clinical testing and movement towards the control of TB.

The rational design of highly attenuated strains that do not cause illness is possible, although a sustained investment in TB basic research to fill the vaccine pipeline is needed.

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