

Although childhood TB accounts for a substantial proportion of the disease burden, it is still neglected because of the difficulty in diagnosis, the unknown outcome of the disease in children and the general perception that childhood TB is less important



Where do they all come from? – Anne Rook
Wood, polycarbonate, 1,500 children's shoes
(3 x 3 x 3 m)

'Who will save this little boy,
smaller than a grain of oat?
Where will the hammer come from,
executioner of this chain?'

The Plowboy (Poem) – Miguel Hernández

Anne Rook
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Anne Rook has exhibited extensively in Britain, France, and Japan. She has also produced several artists' books, a number of which are in the Victoria and Albert Museum, London. Among her publications are: *Prints Now*, Gill Saunders and Rosie Miles, V & A Publications, 2006; and 'Sunek/Thrust', 26th Biennial of Graphic Arts, Ljubljana, 2005.

In 2000 she was commissioned to produce an outdoor sculpture for 'Riverside' East International Norwich.

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Attenuated Vaccines

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Today live vaccines are considered the cornerstone of all new TB vaccine approaches; from prime-boosting to improve BCG or as a replacement for BCG, attenuated MTB strains are regarded as reliable pre-exposure vaccines that could replace BCG. The development of mycobacterial genetic tools and genomics in the last 20 years and the advances in our understanding of TB immunopathogenesis have renewed optimism of developing new prophylactic vaccines conferring better protection than BCG in the coming years.

This chapter describes the state-of-the-art of attenuated live vaccines candidates. Many candidates have undergone the required progressive development from discovery, to preclinical testing on safety, and protection against TB. Promising live vaccine candidates are ready to enter Phase I clinical trials and these are extensively reviewed. Safe and effective rationally attenuated MTB strains are potential candidates to substitute BCG in the near future.

The Need for a Better Vaccine than BCG against Pulmonary TB

Prophylactic vaccines are one of the most useful and cost-effective tools for reducing morbidity and mortality associated with infectious diseases (1). New, and still unmet, targets for vaccine development include some of the more difficult infectious agents, such as HIV, MTB, and parasitic diseases like malaria (2), the ‘big three’ killer infectious diseases which cause major public health

problems. In contrast to AIDS and malaria, there is a vaccine in use today for the prevention of TB in humans that could be used as a gold standard for testing improved vaccine candidates.

BCG was developed nearly a century ago as an attenuated live vaccine for TB control. The original BCG vaccine strain was derived from a clinical isolate, *M. bovis*, which causes TB in cows. Although BCG is the most widely used vaccine in human history, the mechanisms of attenuation are only starting to be understood (3). From 1921, BCG was generously distributed worldwide between microbiologists, resulting in the evolution of a number of daughter strains. It was not until 1960 that BCG was freeze-dried and subsequently adopted as the primary seed lot (4). During the subcultivation process that led to its attenuation, BCG has lost more than 100 genes that are present in MTB (5). Subculture of the original BCG strain in different laboratories resulted in BCG being no longer a single organism but comprising a number of substrains that differ in their genotype and phenotype with different immunological properties (6).

Comparative genomic studies showed that all BCG vaccines lack RD1, the region implicated in virulence due to the presence, among others, of the important immunodominant ESAT6 antigen (4). The 1173P2 BCG Pasteur strain has been sequenced (7). It is important to note that five of the six immunodominant antigens of MTB (ESAT-6, CFP10, Ag85, MPB64, MPB70, and MPB83) are either deleted or down-regulated in some or all BCG strains (5). The molecular mechanisms that contribute to the attenuation of BCG substrains include natural mutants of major virulence factors of MTB such as the ESAT6 antigen, the virulence lipids phthiocerol dimycocerosate (PDIM) and phenolic glycolipid (PGL), and the transcription factor (PhoP). BCG strains differ markedly in their degree of virulence that could account for the differences in protective efficacy and tuberculin reactivity or adverse reactions between the different strains (3, 4, 8). The study and understanding of these new insights have extremely important implications for the development of future vaccines.

The systematic use of BCG is responsible for saving thousands of lives each year, but its benefits seem to be restricted to the prevention of severe childhood forms of the disease, including miliary and extrapulmonary TB and the often fatal TB meningitis. For this reason, BCG vaccination is recommended and included in the calendar of vaccination by WHO in countries with high incidence of TB. The 100 million BCG vaccinations given to infants in 2002 will have prevented 30,000 TB meningitis cases in children during their first five years of life, and about 11,000 cases of disseminated or miliary TB (9). Unfortunately, in adults, the protective efficacy of BCG against pulmonary forms of TB, the main transmissible form of the disease, is variable and in many cases

inefficient (6, 10). Consequently, a new TB vaccine conferring better protection against the pulmonary manifestations of the disease is urgently required to reduce the incidence of TB in endemic areas.

Live Attenuated Vaccines as an Alternative to BCG

Classical vaccine candidates have to mimic natural infection as closely as possible without causing disease (11). Consistent with the evolution of the immune response to provide protection against infectious diseases, the optimal development of a protective immune response by a vaccine should reproduce the steps and processes elicited during the establishment of natural immunity (2). Epidemiological and animal studies indicate that previous infection with TB confers relative protection against subsequent disease due to re-exposure (12, 13). This suggests that attenuated live vaccines that will not cause disease could elicit the development of protection against TB. The rational design of highly attenuated strains that do not cause illness is today possible and the utility of live vaccines to induce a strong and long-lived immunity against intracellular pathogens has been widely proven (14, 15).

The MTB H37Rv and H37Ra strains are widely used in laboratories as reference standards. Both strains were obtained in 1934 by serial passage of an MTB strain named H37 that was originally isolated from a TB patient in 1905. Subcultivation resulted in segregation of a virulent strain (H37Rv), the first MTB strain sequenced (16), and an avirulent strain (H37Ra). Because of its stable attenuation, H37Ra has been widely used in many laboratories in the world since 1934. It was, indeed, the first well-documented MTB attenuated strain used as an experimental TB vaccine in different animal models. Early experimental studies in guinea pigs showed that at equivalent dose, H37Ra has about the same sensitizing potency as that of BCG and was equally effective in prolonging survival time (17). Furthermore, experiments in mice showed that H37Ra was as effective as BCG in producing immunity against aerogenic infection with virulent TB bacilli. The immunizing capacity of H37Ra and BCG were approximately the same. Immunization with 10 to 20 x 10³ organisms (BCG or H37Ra) protected 50 per cent of animals against development of pulmonary lesions when challenged with aerosolized H37Rv (18).

Despite the long use of H37Ra, the mechanisms of attenuation are only recently being understood. The sequence of H37Ra strain has been published (19, 20, 21). A point mutation in the transcriptional regulator *phoP* is partially involved in the attenuation of H37Ra via three mechanisms: (a) lack of secretion of the major T cell antigen ESAT-6 (20); (b) loss of production of

polyketide-derived acyltrehaloses comprising sulpholipids (SL), diacyltrehaloses (DAT), and polyacyltrealoses (PAT), all of them implicated in immunoregulation (22) and; (c) transcriptional differences between H37Rv and H37Ra (23, 24). The strong attenuation of the H37Ra strain is likely based on other mutations that contributed to the loss of important MTB factors such as PDIM which, in combination with the *phoP* mutation, resulted in complete attenuation of H37Ra. The genetic basis of the loss of PDIM in H37Ra remains to be elucidated (22).

The rational design of potential attenuated live vaccines against TB is likely to benefit from over 80 years of experience in the use of BCG. 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 make live vaccines as highly valuable potential candidates for combating TB (25, 26, 27). One of the advantages of using attenuated MTB strains as vaccine candidates is that major antigens would still be retained, thus providing a rational solution for replacing BCG.

The essential criteria for the construction of new attenuated MTB strains to be used as vaccine candidates would include the rational design and a profound molecular characterization coupled with stability as well as immunological and safety studies. It is also requisite to compare a potential live attenuated MTB with present vaccine BCG in different relevant animal models such as guinea pigs (a very susceptible host) for protection assays and immunocompromised mice for safety studies (28).

The Development of Mycobacterial Genetics

The improvement of live attenuated vaccines has often been limited by a lack of genetic tools in mycobacteria. Pioneering studies performed in Europe by Brigitte Gicquel's group (29, 30) and in USA by William Jacobs' group (31, 32) provided some essential mycobacterial genetic tools that are used today. Mycobacterial genetics was initially developed in the fast-growing mycobacteria (*mc*²155) and later in slow growing mycobacteria, namely BCG (an attenuated non-pathogenic model) and MTB.

Systematic studies of genes involved in virulence in mice, based on the use of a random signature-tagged transposition library, resulted in the identification of 16 attenuated mutants (33, 34). The systematic transposition of MTB allowed researchers to map the genes that are essential for MTB to grow (35). In the last decade, the molecular methods for inactivating selected genes have made it possible to attenuate MTB strains in a rational manner (36–38); this together with the availability of the genome sequence have led to the construction of defined

mutants (16) allowing researchers to analyse the contribution of individual genes to MTB virulence. This has made it possible for us to construct well-defined attenuated MTB strains that could be tested latter as potential vaccine candidates in different animal models in a step-wise manner (25, 27, 39).

Discovery of New Attenuated Live Vaccine Candidates

There are many potential target genes in MTB that could be inactivated and the mutants could be assessed as potential new vaccines. One of the most difficult questions when designing a strategy for attenuating a clinical isolate of MTB is the choice of gene or genes to be targeted from the around 4,000 genes in the genome (16). Different approaches have been taken for the construction of rational attenuated MTB.

Auxotrophic Mutants of Amino Acid or Nucleic Acid Synthesis

The elimination of genes that are essential for amino acid or nucleic acid biosynthesis would only allow growth of the bacteria in the presence of selected nutrients. The genes implicated in this biosynthesis are potential targets for the construction of mutants that will only facilitate growth of the mutant MTB in appropriated conditions in the laboratory when the media is supplemented with specific nutrients. The bacteria will not be able to survive in eukaryotic cells where the nutrient is not available, and would only have limited replication cycles within the host. Such mutants will display various degrees of attenuation and have diverse potential as vaccine candidates to be tested in animal models (39).

Auxotrophic mutant MTB strains constructed using allelic exchange to disrupt proline (*proC*) and tryptophan (*trpD*) biosynthetic genes have previously been described (40). MTB *proC* and *trpD* mutants were highly attenuated following infection of murine bone marrow-derived macrophages and in the SCID mice. Auxotrophic mutants of *proC* and *trpD* conferred protection in mice challenged with MTB H37Rv. However, when *trpD* mutant was tested in guinea pigs, the protection conferred was no better than BCG (41).

Another example of auxotrophic mutant construct of MTB was the disruption of the purine biosynthetic gene, *purC*. The mutant was attenuated when tested in vitro, where it was unable to grow within mouse bone marrow-derived macrophages, and in vivo in mouse and guinea pig models of low dose infection by aerosols (42). In addition, the mutant induced strong DTH response to purified mycobacterial protein antigens and conferred some protection in guinea pigs.

MTB *purC* mutant showed a protective effect in the lungs but not in the spleen, measured as bacilli loads, which could reflect the inability of the MTB auxotroph to control the dissemination of MTB H37Rv after low dose aerosol challenge, probably because it was unable to multiply within the host-infected cells, producing poor stimulation of protective immunity.

Thus, it seems that the attenuated mutant strains need to retain a limited ability to grow and disseminate within the host in order to induce protective immunity. As Rene Dubos hypothesized, a not-too-low residual virulence is an important attribute of BCG and there were different *in vitro* and *in vivo* characteristics of several substrains of BCG affecting their protective efficacy which can be applicable to new attenuated vaccine candidates (8, 43). Indeed it has been demonstrated that only MTB-attenuated strains, which can multiply during the immunization period, conferred protection to vaccinated animals (39). This is why, for intracellular pathogens, vaccines made with dead pathogens tend to be less effective than live attenuated organisms (44).

Auxotrophic Mutants of Lipid Synthesis

One important characteristic of MTB is the high lipid content of its cell envelope (45). Lipids constitute up to 40 per cent of MTB dry weight. Lipids biosynthesis and metabolism in MTB has been implicated in virulence and interference with the immune system of the host, and they are crucial in the mycobacteria intracellular replication and persistence (22, 46–49).

Pantothenic acid (vitamin B5) is an essential molecule for the synthesis of coenzyme A and acyl-carrier proteins; both are important molecules in fatty acid metabolism and biosynthesis of polyketides, among other metabolic reactions. A double-deletion mutant of MTB in the *panC* and *panD* genes involved in the pantothenate synthesis has been constructed (50). This auxotrophic mutant is attenuated when tested in BALB/c and SCID mice, and conferred protection when used as subcutaneous vaccine in mice challenged with low aerosol doses of virulent H37Rv. Construction of double auxotrophic mutants has been described in order to increase attenuation and stability of the attenuated phenotype and they also include deletion of virulence regions such as RD1 (51). 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 (50). Protection levels equivalent to BCG were generated in the lungs and spleen of vaccinated guinea pigs, with reduced dissemination of infection to the spleen at five weeks after aerosol challenge with MTB (52).

Mycobacterial Lipids Implicated in Virulence

PDIM are a lipid family of the external mycobacterial cell wall (53) that are produced by the combined action of fatty acid synthases and polyketide

synthases (PKS). The *FadD26* protein synthesizes acyl-adenylates of long-chain fatty acids. In this way, these fatty acids are activated and transferred to the cognate PKS proteins permitting subsequent extension, incorporation, and production of PDIMs (54). PDIMs are physiologically important molecules for mycobacteria because they are involved in cell wall permeability and in virulence (33, 34, 55). The immunogenicity and protective efficacy of the MTB *fadD26* mutant, which depicts impaired synthesis of PDIM, has been demonstrated (56). Virulence studies in BALB/c mice confirmed its attenuation with larger DTH response and lower but progressive production of IFN- γ and TNF- α than its parental strain. The *fadD26* mutant given subcutaneously induced a higher level of protection than BCG when challenged with the Beijing strain of MTB. Similarly, there was less tissue damage (pneumonia) and lower CFU in the mice vaccinated with the *fadD26* mutant as compared to those of BCG-vaccinated mice. These data suggest that inactivation of PDIM synthesis can increase immunogenicity and prolong protection.

Other Mycobacterial Genes

Proteins coded by the mammalian cell entry (*mce*) genes allow for cell invasion into the host (57). The MTB *mce-2* and *mce-3* mutants were attenuated in BALB/c mice (58). Infection of BALB/c mice with a high dose of any of these two *mce* mutants induced lower but progressive production of IFN- γ and TNF- α , as well as larger DTH reactions, than their parental H37Rv strain (59). Both mutants were more attenuated than BCG in both immunocompetent BALB/c mice and immunodeficient nude mice. Cell suspensions from lymph nodes and spleens of *mce* mutant vaccinated mice stimulated with mycobacterial culture filtrate antigens or immunodominant antigens (ESAT-6, Ag85) produced more IFN γ compared to BCG-vaccinated animals. Used as subcutaneous vaccines, 60 days before intratracheal challenge with H37Rv or the hypervirulent strain of MTB (Beijing), both *mce-2* and *mce-3* mutants induced higher levels of protection compared to BCG. These data suggest that the lack of *mce-2* and *mce-3* gene expression decreases virulence and increases immunogenicity of live vaccines, favouring their ability to protect against TB.

Another strategy to rationally attenuate MTB involves inactivation of *secA*. This gene encodes a component of a mycobacterial protein secretion system involved in inhibiting apoptosis of infected cells and, consequently, promoting MTB survival within the host. Conversely, inactivation of *secA* results in increased host cell apoptosis and increased 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 currently in preclinical development (60).

Another approach is the inactivation of transcription factors essential for MTB virulence such as *phoP*. Its inactivation confers full-attenuated phenotype

as determined in cellular and animal models (61). This mutant will be extensively described in the next section.

More Developed New Live Vaccine Candidates

PhoP: A Major Regulator of Complex Virulence Network in MTB

The *phoP* gene corresponds to Rv0757 in the MTB genome (16) and was reported to encode the transcription factor of the PhoP/R two-component system (TCS). TCS enable 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 (16, 62), 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. PhoP was firstly implicated in mycobacterial virulence since a remarkable increase in the *phoP* gene expression was observed in *M. bovis* strain (B strain) (63). The B strain was first isolated in 1991 and was responsible for the deaths of more than a hundred people in an outbreak largely confined to individuals coinfecting with HIV (64, 65). Unlike other strains of *M. bovis*, the B strain is transmitted between humans through respiration, resulting in high rates of reinfection in TB patients (66).

PhoP/R was found to play an essential role in MTB virulence since *phoP* inactivation conferred an extremely attenuated phenotype in mice macrophages. (67) When the *phoP* gene was disrupted by the insertion of a kanamycin resistance cassette in the MT103 strain, a fully virulent MTB clinical isolate, the resulting mutant (named SO2) (67) displayed marked changes in bacterial and colony morphology, together with impaired multiplication in mouse macrophages in vitro. SO2 has been found to be attenuated in vivo, in a mouse infection model, in which it persisted in the organs but was unable to replicate (24). In addition, this *phoP* mutant displayed major modifications in the lipid composition of the MTB cell envelope, given that PhoP positively regulates the synthesis of complex lipids, such as SL, DAT, and PAT (68). These complex lipids are considered as important virulence factors (69) with immunomodulatory effects on human CD4+ and CD8+ T cells (46, 70), thus interfering with the immune system of the host. These phenotypes support the results obtained by microarray studies in which a set of genes potentially regulated by PhoP were identified (24, 71). These microarray data also show decreased expression of other important virulence factors previously described as *lipF*, *fbpA*, and *mmpL8*

(34), confirming the importance of PhoP-regulated genes in MTB virulence. Additionally, as described previously, a point mutation in *phoP* account for the avirulence of the H37Ra strain (20, 22, 72) demonstrating that PhoP is a global regulator of key functions required for the successful intracellular survival of MTB within host cells.

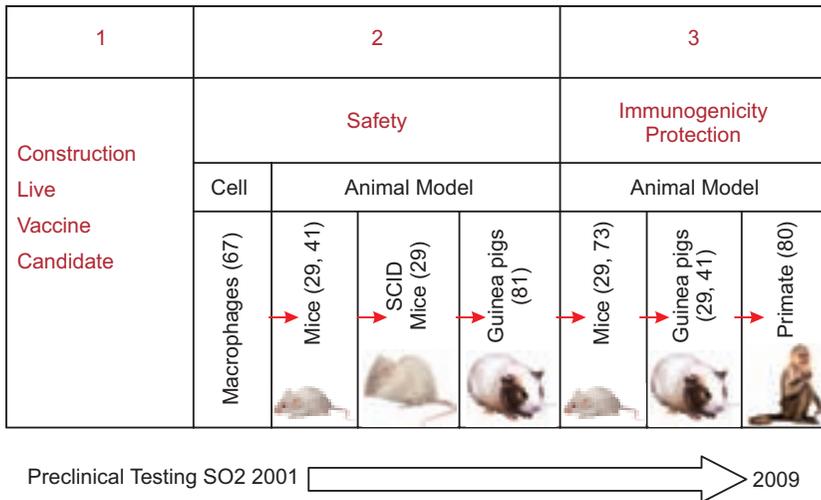
Since PhoP is an essential regulator of virulence network in MTB, we constructed an MTB *phoP* mutant to determine its potential as an attenuated live vaccine candidate.

Preclinical Protection Studies of Live Vaccines Based on *phoP*

In order to demonstrate proof of principle of live vaccine based on *phoP* inactivation, its potential as a prophylactic vaccine against challenge with MTB has been studied in various animal models. We used the clinical MTB MT103 strain, rather than the reference strain H37Rv, to avoid the need for subculture in the laboratory which might lead to substrain variability. The *phoP* gene disrupted strain, designated SO2, has been previously described and was tested as a prototype vaccine in preclinical studies conducted between 2001 and 2009 (61, 67, 73).

The protective efficacy of SO2 was systematically studied in relevant animal models (Figure 13.1). The results of BALB/c mouse vaccination experiments

Figure 13.1 Live Vaccine Testing



Note: Step-by-step preclinical testing strategy on safety and protection against tuberculosis for attenuated live vaccines based on Douglas Young, 'Road Map'.

indicated that SO2 had vaccine characteristics similar to those of BCG Pasteur (61, 73). Cellular immunity is considered particularly important for protection against TB (74). We therefore quantified CD4⁺ and CD8⁺ specific responses in BALB/c mice vaccinated with SO2 and compared 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 compared to BCG-vaccinated animals (61).

T helper cells play a key role in immune responses to MTB infection because they activate macrophages, kill intracellular MTB, and promote the development of cytotoxic T lymphocytes, which kills infected target cells (47). The Th1 subtype is responsible for cell-mediated immunity and produces IFN- γ and IL-2 as the major cytokines protecting against TB (47). The severity of TB disease is strongly correlated with IL-4 levels, this cytokine working in concert with TNF- α 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, the latter was found to induce lower levels of IL-4 in the lungs of infected mice and associated with a lower percentage of lungs affected by pneumonia. Levels of IFN- γ , IL-4, and TNF- α were higher in the lungs of MT103-infected mice, which displayed progressive disease and greater inflammation than SO2-infected animals (73). In the case of SO2 infection in mice, protective immunological responses were accompanied by a decrease in the bacterial load in the lungs and lower levels of dissemination to other organs, indicating the potential of SO2 as a protective prophylactic vaccine.

Although the mouse model provides valuable information about a number of immunological parameters, guinea pigs are widely recognized to constitute a more susceptible model of TB, with many similarities in the progression and pathology of the human disease (76, 77, 78). The two models represent different points on a scale of susceptibility; both models have their advantages but the guinea pig model is used as the disease susceptibility model which makes it a stringent model for vaccine screening (76, 77, 78). Within the European TBVAC consortium, various vaccine candidates have been compared in the guinea pig model of infection (41). Animals were immunized by aerosol and challenged with a high dose of MTB. SO2, at single dose, and BCG boosted with MVA and the immunodominant antigen Ag85A, were 2 of the 24 TB vaccine candidates tested that conferred greater protection than BCG. Survival was significantly longer in guinea pigs vaccinated with a single dose of SO2 than in animals vaccinated with BCG (41). 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 (61). Thus, this vaccine candidate displayed generally good parameters of protection: prolonged survival, lower bacterial load in organs, and fewer lesions.

These encouraging findings in the guinea pig model led to further studies in relevant non-human primate models. SO2 administered as a single dose and MVA85A expressing Ag85A as boost after BCG prime vaccination (79) were studied in the non-human primate model (80). SO2 showed significant protective efficacy by various parameters in rhesus macaques. SO2 was well tolerated and induced specific IFN- γ responses to MTB proteins. After challenge with high dose of MTB Erdman, SO2 vaccinated animals showed reduced average lung bacterial counts and reduced lung pathology. Significant protective effect as displayed by reduction in either body weight loss or C-reactive protein levels and other haematologic parameters as markers of inflammatory infection were observed compared to non-vaccinate controls (80).

Safety Studies of Live Vaccines Based on *phoP* Mutation

Safety is one of the main concerns and a major challenge for attenuated live vaccines in order to provide enough assurance for its future use in humans. Survival studies in immunocompetent BALB/c mice showed the SO2 mutant to be fully attenuated with respect to wild-type MTB (73). Survival studies in immunocompromised SCID mice infected with bacterial aerosols showed that animals infected with MTB died within 40 days of infection, whereas animals infected with the *phoP* mutant survived to the end of the experiment (6 months), and no bacteria were recovered from the lungs or spleens of these animals (61). Additional survival studies in the SCID mouse model intravenously infected with different doses of SO2 demonstrated this mutant to be even more attenuated than BCG (61).

Since rearrangements in the *phoP* mutation could happen in a live vaccine after subcultivation, the stability of this mutation after successive passages in culture media was studied. Results indicated no loss of the kanamycin resistance cassette in the six months of the study. Additional *in vivo* studies showed that three months after intravenous inoculation of SO2 in immunocompromised SCID mice, the kanamycin resistant phenotype was conserved. These data indicate that insertion of the antibiotic cassette within the *phoP* gene was genetically stable during the period of experiment, both *in vitro* and *in vivo* (81).

Another important question that arises is whether after genetic modification of MTB 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 wild type MTB and this could be due to changes in the cell

envelope of the *phoP* mutant (67, 68). All these results indicate that the SO2 strain is sensitive to major antituberculous drugs and, in case of infection with SO2-based vaccines, it would be possible to use such drugs to treat it (81).

Toxicity testing in guinea pigs by using high doses of vaccine candidates is a standard test for the absence of virulence in BCG and should be an important requirement for the validation of new live vaccines. In this study, animals were inoculated with 2.5×10^6 CFUs of SO2 (50x the standard vaccination dose in guinea pigs). Results 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 six months follow up (81). Determination of DTH in guinea pigs at the end of this study reflected similar values to the ones obtained four (83) or six weeks (76) after immunization with BCG, thus reflecting the conservation of the immune response.

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' (84). A number of vaccine candidates have recently been tested to assess the effectiveness and lack of toxicity after post-exposure vaccination (84). 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 (85, 86) to address this question, and we can conclude that no toxic effects have been developed in either case, 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 (81). In any case, the lack of toxicity in these models gives us an idea of how safe this vaccine is, and may probably be safe to be used in subjects with latent TB infection.

Taken together these safety studies encourage the use of the SO2 strain as a starting point for the construction of the next generation of attenuated live vaccines

Safety and Regulatory Challenges to the Use of Live Organisms

Safety is a key concern in the use of live vaccine candidates. Candidate attenuated live vaccines require non-revertible genetic mutations that affect the virulence of MTB but would still allow the induction of an adaptive immune response

capable of protecting against TB disease. Safety and regulatory obstacles to the use of live organisms must still be overcome. 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, more than 25 per cent of the 250 or so children vaccinated with a particular batch of BCG in Lubeck (Germany) developed TB. It was later recognized that this batch had been accidentally contaminated with a virulent strain of MTB (87).

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 (88). Therefore, attenuated MTB vaccines with increased safety features are of extraordinary importance in order to decrease the risk of vaccine dissemination in HIV-infected recipients (88). The risk of disseminated BCG disease is increased several hundredfold in HIV-infected infants compared to those 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 HIV-infected infants.

For live vaccines based on attenuated MTB, a consensus document was developed in 2004 at a conference in Geneva and the presence of at least two non-reverting independent mutations in the mycobacterial genome was recommended in order to avoid reversion to virulence (89). Regulatory issues are fundamental for the development of new TB vaccines (90).

Conclusion

After the Geneva Consensus (89), there is renewed optimism concerning the use of live attenuated vaccines as reliable candidates to enter Phase I clinical trials in the coming years. The development of a new TB vaccine is an integral element of the Global Partnership to Stop TB, a network of international organizations, countries, public and private sector donors, governmental and non-governmental organizations and individuals, which aims to develop a safe, effective, licensed vaccine, available at reasonable cost, by 2015.

If the goal of having an effective licensed vaccine 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 three-four being evaluated in Phase III efficacy trials (9). Advances in TB research have made this goal possible. 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. BCG prime-boost

regimens have been the first candidates to be assessed (79, 91). Recently, two recombinant BCG vaccines have entered Phase I trials in humans (92) (S. Kaufmann—personal communication).

A better knowledge of the virulence regulation in MTB and the immune responses that provide protective immunity have paved the way for a new generation of potential live vaccines. Protection studies performed in diverse animal models have shown that some MTB mutants are superior to BCG. Viability, persistence, and high immunogenicity are key attributes required for a successful TB vaccine based on live bacilli in order to confer an appropriate level of protection. Rational attenuated live vaccines are considered today, promising candidates with the enormous potential to replace the present BCG vaccine or to be used as a priming vaccine in future prime boost strategies.

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