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The dream of a vaccine against tuberculosis; new vaccines improving or replacing BCG?

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ABSTRACT: In the last 10 yrs, work with experimental laboratory models has developed many new vaccine candidates against tuberculosis (TB). They include subunit vaccines, modified bacilli Calmette-Guérin (BCG) and attenuated *Mycobacterium tuberculosis*. Phase I clinical trials of new TB vaccine candidates have begun for the first time after 80 yrs of use of BCG. Many of these new trials involve recombinant BCG or improve BCG immunity by boosting with vaccines consisting of subunits or attenuated vaccinia virus expressing TB antigens.

However, effective vaccination against TB presents diverse and complex challenges. For example, TB infection can become reactivated years later and infection does not guarantee resistance to a subsequent second infection. A truly effective TB vaccine may, therefore, have to elicit an immune response that is greater than that induced by natural infection. In addition, various different populations have to be protected including those vaccinated with BCG and those infected with *M. tuberculosis* or HIV.

The goal is a new generation of vaccines effective against respiratory forms of tuberculosis. As a first step, good candidate vaccines able to boost bacille Calmette-Guérin and thereby improve protection could be a reality in the near future. Tuberculosis vaccine candidates, able to replace the currently used bacille Calmette-Guérin and/or make the eradication of tuberculosis feasible, can only be expected in the long-term, and safe live vaccines could be promising candidates.

KEYWORDS: Live vaccines, new vaccines, pre-clinical and clinical trials, tuberculosis

Mycobacterium tuberculosis is responsible for more deaths than any other single infectious organism; there are >7 million new cases and 2 million deaths annually. Control strategies for tuberculosis (TB) rely heavily on case detection and treatment with at least three different drugs for long periods of time. Consequently, the development of multidrug resistance is a serious impediment to any attempt to control this disease [1]. No new drugs have been added to the first-line treatment regimen for TB for >30 yrs. In addition, the public health impact of *M. tuberculosis* has become increasingly severe, partly because of the HIV epidemic. There is a clear synergy between *M. tuberculosis* and HIV, and active TB increases HIV-related immunodeficiency and mortality [2]. Indeed, TB remains the largest attributable cause of death in HIV-infected individuals and is responsible for 32% of the deaths of HIV-infected individuals in Africa. The neediest populations, in countries where TB incidence is highest, do not have access to treatment and, furthermore, in many cases, anti-TB drugs are ineffective. The development of an effective TB vaccine is obviously now an urgent priority. Given the variable protective efficacy generated by BCG vaccine against TB, there is a

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concerted effort worldwide to develop better vaccines that could be used to reduce the burden of TB (table 1).

THE CURRENT VACCINE AGAINST TB: BCG

The current TB vaccine, bacillus Calmette-Guérin (BCG), is a live vaccine that protects against severe childhood forms of disease, including milliary and extrapulmonary TB and the often fatal TB meningitis. It also confers protection against leprosy. The World Health Organization (WHO) recommends BCG vaccination in areas of high TB prevalence and incidence. BCG vaccination is currently compulsory in $\geqslant$64 countries and administered in >167 countries [3, 4]. Indeed, BCG remains the most widely used vaccine in the world.

BCG is an inexpensive vaccine that has been given to >2.5 billion people since 1948. It has a long-established safety profile and has outstanding adjuvant activity, eliciting both humoral and cell-mediated immune responses. It can be given at birth or any time thereafter and a single dose can produce long-lasting immunity. It has also been licensed as a treatment for bladder cancer.

Recently, an investigation of the long-term efficacy of BCG vaccine, a 60-yr follow-up study in American Indians and Alaska natives, has shown remarkable results that the efficacy of BCG vaccine persists for 50–60 yrs, suggesting that a single dose of BCG vaccine can give life-long protection [5].

Complications of BCG immunisation among children with HIV infection are very rare. The risk of disseminated BCG among adult AIDS patients with childhood BCG immunisation is very low, and, in addition, childhood BCG immunisation is associated with protection of adults with advanced AIDS against bacteremia with *M. tuberculosis*. Studies in Zambia have shown that bacteriemia due to BCG or *M. tuberculosis* is rare among children with BCG immunisation (even recent) and symptomatic HIV infection [6].

The level of protection conferred by BCG is extremely variable; it differs according to the form of pulmonary TB and can be affected in those cases in which TB is associated with AIDS.

TABLE 1 Major challenges and concerns for tuberculosis (TB) vaccine development

Challenge	Concern
Infection with *M. tuberculosis* does not confer protective immunity against TB	Degree of protection: new TB vaccines should confer a stronger immune response than *M. tuberculosis* infection
One-third of human population infected with TB	Preventive and immunotherapeutic vaccines are necessary
Co-infection HIV/TB	Safety: vaccines with at least the same degree of attenuation or more than BCG are needed
Large percentage of the population vaccinated with BCG	New vaccine candidates should be tested in the BCG-vaccinated population

M. tuberculosis: Mycobacterium tuberculosis; BCG: bacillus Calmette-Guérin.

The efficacy of BCG vaccines against pulmonary TB varies between populations, showing no protection in Malawi but 50–80% protection in the UK [7]. The reasons for the failure of BCG have been widely debated, and remain a topic of active research. Natural exposure to environmental mycobacteria is thought to exert an important influence on the immune response, and this may mask or otherwise inhibit the effect of BCG vaccination in tropical countries. This type of phenomenon has been proposed as a plausible explanation for the north-south gradient in the effectiveness of BCG [8].

GENETIC DIVERSITY BETWEEN BCG VACCINES

The current BCG vaccine was obtained from a pathogenic clinical isolate of *M. bovis* and attenuated in the laboratory by 230 serial passages on potato-glycerine-ox bile medium between 1908–1921. One of the various reasons proposed to explain the variable efficacy is the diversity of BCG strains. It is now clear that, after many years of growth and passaging in the laboratory, divergent BCG vaccine strains have evolved, which differ from each other and from the original BCG. From 1921, when BCG was first used, to 1961 when WHO recommended the lyophilisation and storage at -80℃ for BCG cultures, vaccine BCG was subcultured by numerous laboratories worldwide. As a consequence, numerous variants appeared, including BCG Pasteur, BCG Moscow and BCG Brazil [9]. These various BCG strains are different from each other and from their ancestors, such that it is prudent to refer to BCG vaccines in the plural due to differences in protection and effectiveness. [10].

BCG vaccines have been classified into two major groups. BCG Tokyo, Moreau, Russia and Sweden secrete a lot of MPB70, have two copies of the insertion sequence IS*6110*, and contain methoxymycolate and MPB64 genes. In contrast, BCG Pasteur, Copenhagen, Glaxo and Tice secrete little MPB70, have a single copy of the insertion sequence IS*6110*, and do not contain the methoxymycolate and MPB64 genes [11].

Comparative genomic analysis has revealed the existence of several *M. tuberculosis*-specific regions that have been deleted from BCG. Genomic comparisons have made it possible to determine the order of genetic events. These events include deletions and duplications, and changes in IS*6110* copy number that occurred between its first use in 1921 and 1961 [9]. These complex genomic rearrangements in BCG strains have undoubtedly led to phenotypic and immunological differences and may contribute to the variability in vaccine efficacy. All these points reinforce the requirement for vaccines that are more effective than the currently used BCG vaccines against the respiratory forms and that are able to eradicate TB. Problems of substrain variability and protective efficacy of the current BCG vaccines could be overcome by new rationally constructed live vaccines, for which the attenuation factor and immunity are known.

THE IMMUNE RESPONSE AGAINST TB

The lung is the portal of entry of *M. tuberculosis* in most human infections and provides a suitable environment for this slowly replicating pathogen. The infection is established in alveolar macrophages of the distal alveoli before it is recognised by the adaptive immune response 5–6 weeks later. CD4+ and CD8+ T cells are recruited through the lung, inducing protective immunity.

Both CD4+ and CD8+ T cells are essential for protective immunity against *M. tuberculosis*. Resistance to *M. tuberculosis* involves the activation of mycobacterial-specific CD4+ and CD8+ T cells by dendritic cells (DC), which migrate from the site of the infection in the alveoli to the draining lymph nodes. The development of interferon (IFN)-γ-secreting CD4 T cells is dependent on the secretion of interleukin (IL)-12 by infected DC. Subjects deficient in receptors for IFN-γ and IL-12 are extremely susceptible to mycobacterial infections, confirming the absolute requirement for T-helper cell type 1 (Th1)-like T cells for host immunity [12].

The nature of an effective immune response to TB is incompletely understood, but the most effective vaccination strategies in animal models are those that stimulate T-cell responses, both CD4 and CD8, to produce Th1-associated cytokines. Therefore, formulations that induce the production of enduring Th1 responses are desirable, and doubtless an essential element of a successful vaccine. Several adjuvant or live vaccines capable of inducing potent T-cell responses have been developed and some have entered clinical testing.

CHALLENGES FOR TB VACCINE DEVELOPMENT

There are a number of substantial underlying problems to be faced in developing vaccines with enhanced protective efficacy against TB. In contrast to a classical vaccine-preventable disease such as smallpox, recovery from infection with *M. tuberculosis* is not associated with sterilising immunity against reinfection after clearance of the original infection with antibiotics. Studies of the molecular epidemiology of TB indicate that reinfection with new strains of TB is more frequent than previously believed [13]. Therefore, vaccines need to be more effective than infection with *M. tuberculosis* itself.

One-third of the population worldwide is estimated to be infected with *M. tuberculosis*, and therefore any new TB vaccine should be suitable for use in subjects pre-exposure, to prevent infection, but also post-exposure, to prevent the development of disease or as an immuno-therapeutic agent to act with antimicrobials to increase the rate of clearance of *M. tuberculosis*.

An additional challenge is that as a large percentage of the human population has already been immunised with BCG, and so any new generation vaccines against TB must also be able to protect the population that has already been vaccinated with BCG. Obviously, new vaccines must also be safe enough to be used in HIV-infected individuals [14].

RESEARCH FOR NEW VACCINES AGAINST TB

Advances in the field of immunology, investigations with vaccines and technological developments have provided insights into the genetics of the TB bacillus [15]. This puts researchers in a better position for the construction of new effective and safe vaccines against TB. A large number of groups in numerous countries have embarked on the ambitious project of finding new vaccines that provide a greater level of protection than the present BCG [16, 17]. A large number of vaccine candidates have been proposed as a result of the basic research during the last decade.

Broadly, two approaches have been used to improve the TB vaccine. The first involves subunit vaccines. However, no viable subunit vaccines have been generated that can deliver immunodominant mycobacterial antigens. Both protein and DNA vaccines induce only partial protection against experimental TB infection in mice, but their efficacy has generally been no better than that of BCG [18]. New antigen formulations, including multiple antigens or epitopes, are under investigation and it is hoped that they will afford better protection in humans [19, 20]. The second approach involves using live vaccines. These may be BCG strains that have been genetically manipulated to express immunodominant antigens, or attenuated strains of *M. tuberculosis* produced by random mutagenesis and targeted deletion of virulence genes [21].

SUBUNIT VACCINE CANDIDATES

Results with nonviable subunit vaccines are encouraging: their protective effects have to be at least equivalent to those with BCG before they can be considered for human trials. Subunit vaccines have been selected by various rational and experimental approaches.

Potential TB subunit vaccines have been obtained by using immunodominant TB antigens, as in the case of ESAT-6, which confers some degree of protection against *M. tuberculosis* in mice [22]. Protein fusions based on ESAT-6 and antigen 85B administered with a strong adjuvant to mice induce a dose-dependent immune response to the fusion proteins. This immune response was accompanied by protective immunity comparable to BCG-induced protection over a broad dose range. The vaccine-induced efficient immunological memory remained stable 30 weeks post-vaccination [19].

Key *M. tuberculosis* antigens have been identified by analysis of host responses in healthy individuals, and purification of proteins from positive donors. These selected antigens have been used for the development of subunit vaccines against TB, for example Mtb72F, which codes for a 72-kDa polyprotein (Mtb32(C)-Mtb39-Mtb32(N)). Immunisation of mice with Mtb72F protein formulated in the adjuvant AS01B generated a comprehensive and robust immune response, eliciting strong IFN-γ and antibody responses for all three components of the polyprotein vaccine and a strong CD8(+) response directed against the Mtb32(C) epitope. Mtb72F immunisation resulted in the protection of C57BL/6 mice against aerosol challenge with a virulent strain of *M. tuberculosis*. Most importantly, immunisation of guinea pigs with Mtb72F resulted in prolonged survival (>1 yr) after aerosol challenge with virulent *M. tuberculosis* comparable to BCG immunisation. Mtb72F in the AS02A formulation is currently in phase I clinical trials, making it the first recombinant TB vaccine to be tested in humans [23].

BOOSTING BCG VACCINE

Experiments using protein subunits in animals previously vaccinated with BCG (BCG+), and using prime-boost protocols give very good results [24]. These experiments used Ag85A, because it was previously demonstrated that most CD4 T cells accumulating in the lungs of memory-immune mice after challenge recognise this antigen. This vaccine strategy may have applications in the prevention of reactivation TB in the elderly.

Heterologous prime-boost immunisation strategies can evoke powerful T-cell immune responses and may be of value in developing an improved TB vaccine. Enhanced immunogenicity and protective efficacy against *M. tuberculosis* has been demonstrated for BCG after boosting with a recombinant modified vaccinia virus called Ankara. The Ankara recombinant modified vaccinia virus, expressing *M. tuberculosis* Ag85A, strongly boosts (BCG)-induced Ag85A specific CD4(+) and CD8(+) T-cell responses in mice. Protection correlated with the induction of Ag85A-specific, IFN-γ-secreting T cells in lung lymph nodes [25].

RECOMBINANT BCG AS A NEW VACCINE AGAINST TB

Recombinant BCG (rBCG) techniques may be useful for the development of a more effective mycobacterial vaccine than the parental BCG now in use. Various strategies have been used to develop rBCG against mycobacteral diseases. One is based on rBCG producing large amounts of autologous protective antigens; these supplementary antigens are designed to enhance immunity to other BCG antigens by increasing the expression of their genes, as is the case for the immunodominant TB antigens. Recombinant BCG vaccine (rBCG30) expressing and secreting the 30-kDa, major secreted protein of *M. tuberculosis*, also referred to as α-antigen and antigen Ag85B [26], is associated with better host survival after challenge than parental BCG in the highly demanding guinea pig model of pulmonary TB. Animals immunised with rBCG30 and then challenged with an aerosol of a highly virulent strain of *M. tuberculosis* survived significantly longer than animals immunised with conventional BCG [27].

Alternatively, BCG genes that have been lost by deletion from parental *M. bovis* strain and that are important antigens can be restored. An example is the case of ESAT-6 deleted from region RD1 of BCG [28]. Both these approaches are attractive for improving or adding antigens to BCG and could be important in conferring immunity against TB.

A second strategy involves enhancement of the relatively low intrinsic ability of BCG to induce the CD8+ T-cell response [16]. This type of the rBCG has been studied, in particular for whether it alters the permeability of the membranes of phagosomes in host cells. Major histocompatability complex (MHC) class I-restricted CD8+ T cells are believed to play a major role in protection against mycobacterial infection. As BCG persists within the phagosomal space of macrophages after infection, bacterial antigens should be released from phagosomal vacuoles into the cytoplasm of host cells, leading to more pronounced presentation by MHC class I. Listeriolysin (Hly) of *Listeria monocytogenes* is a pore-forming sulphydryl-activated cytolysin. It is essential for the release of *L. monocytogenes* from phagosomal vacuoles into the cytoplasm of host cells, thereby facilitating presentation of antigens by MHC class I molecules. rBCG-secreting biologically active Hly have been constructed [29]. This rBCG improves MHC class I-presentation of co-phagocytosed soluble protein.

In another approach rBCG have been constructed secreting diverse cytokines, including IL-2, IFN-γ and others, in an attempt to enhance the immuno-stimulatory properties of BCG [11].

In addition, a major effort is being made to develop rBCG as a vaccine vehicle capable of simultaneously expressing antigens of numerous pathogens. The aim is the development of an effective rBCG vaccine that is effective against a variety of viral, bacterial and parasitic diseases [11].

ADVANTAGES AND DISADVANTAGES OF ATTENUATED *M. TUBERCULOSIS* AS LIVE VACCINE CANDIDATES

Rational attenuated mutants of *M. tuberculosis* are potential vaccine candidates. The development of biological tools has facilitated genetic manipulation of *M. tuberculosis* [15]. These advances, and the completion of the *M. tuberculosis* genome sequence [30], have facilitated the analysis of the contribution of individual genes to *M. tuberculosis* virulence [31].

The advantage of attenuated *M. tuberculosis* strains as vaccines is that many hundreds of genes have been deleted from BCG, as a consequence of the progressive adaptation of BCG strains to laboratory conditions; these genes are still present in *M. tuberculosis* [9]. Of the six immunodominant antigens of *M. bovis* (ESAT-6, CFP10, Ag85, MPB64, MPB70, MPB83), five are either deleted from or downregulated in some or all BCG strains. RD1 is present in all BCG strains. The deletions include the immunodominant antigens ESAT-6 and CFP10, which have recently been shown to be important for protection against *M. tuberculosis* challenge in the guinea pig model [28].

Several studies have described the development of attenuated strains of *M. tuberculosis*. A *M. tuberculosis phoP* mutant has been constructed by a single gene disruption [32] and exhibits impaired multiplication *in vitro* within mouse cultured macrophages; it is also attenuated *in vivo* in a mouse infection model. Thus, *phoP* might be involved in the regulation of virulence in *M. tuberculosis* and is a promising candidate for vaccines. Auxotrophic mutants are attenuated to different degrees and have diverse potential as vaccine candidates as assessed in animal models [33]. Double auxotrophic mutants have been recently described [34].

Some of these live vaccine candidates elicit protective immune responses similar to that of BCG in mice, and better than BCG in guinea pigs (A. Rawkins, Health Protection Agency, Porton Down, Salisbury, UK, personal communication). These findings are encouraging, and further studies in nonhuman primates should be performed. However, there are major issues associated with the use of live organisms; in particular, safety and regulatory hurdles need to be overcome.

FINAL CONSIDERATIONS: BCG+; A NEW BCG GENERATION VACCINE?

Although the efficacy of the BCG vaccine continues to be discussed, live attenuated BCG is still the only vaccine in use for the prevention of TB in humans. This is because it is effective against the severe forms of TB and its use is preventing a large number of deaths that would otherwise be caused by TB every year.

The choice of the BCG strain to be used for vaccination is a very important issue. It is currently difficult to determine which strain should be used, and further detailed analysis of the genomics and immunogenicity of BCG substrains may provide an answer to this important question. WHO and International Union Against Tuberculosis and Lung Diseases

TABLE 2 New candidate tuberculosis (TB) vaccines

TB vaccine type	Definition	Stage of development	References
Subunit vaccines			
72f	Selected antigens identified from human response	Phase I trial	[23]
85B-ESAT6	Recombinant major antigens	Ready for phase I trial BCG boosting strategy	[19]
DNA vaccines			
85B	Plasmid DNA of Ag85B	Pre-clinical testing	[18]
Viral vector			
MVA-85A	Recombinant modified vaccinia virus Ankara Ag85A	Phase I trial BCG boosting strategy	[25, 37]
Live vaccines			
rBCG30	Recombinant BCG: over-expression of Ag85B	Phase I trial	[26, 27]
BCG:RD1	Recombinant BCG: RD-1 of *M. tuberculosis* introduced	Pre-clinical studies	[28]
rBCG-hly	Recombinant BCG: listeriolysin of *Listeria monocytogenes* introduced	Pre-clinical testing	[29]
***M. tuberculosis phoP* mutant**	Attenuated *M. tuberculosis* clinical isolated by *phoP* deletion	Pre-clinical testing	[32]
***M. tuberculosis* auxotrophic mutant**	Attenuated *M. tuberculosis H37Rv by lysA* and *panCD* deletion	Pre-clinical testing	[34]

BCG: bacille Calmette-Guérin; *M. tuberculosis: Mycobacterium tuberculosis*.

(IUATLD) could then use the BCG substrains giving the best protection, and recommend them for future vaccination worldwide [35].

Research to develop improved TB vaccines seems to be at a decisive time. More than 200 vaccine candidates have been proposed as the result of work over recent years in experimental laboratory models, and some are now approaching clinical testing [25, 36]. The transition from laboratory to clinical trials has a wide range of strategic and technical implications. In particular, facilities and funding need to be provided for the production of any successful vaccine appropriate for clinical use. After the Madrid Conference in March 1995 ''Definition of a coordinated strategy towards a new TB vaccine'' organised by the WHO and IUATLD, a joint effort was established involving diverse governmental organisations in Europe (FP5 and FP6 Framework Programmes) and USA by National Institutes of Health and recently the Aeras Global TB Vaccine Foundation.

For the first time, after 80 yrs of widespread use of BCG, evaluations of new candidates in humans are available, including recombinant vaccine virus (table 2) [37]. The development of a new vaccine conferring better protection than BCG, and able to replace it, nevertheless remains a challenge for the scientific community. In order to eradicate TB, appropriate new vaccines must be found.

Subunit vaccines have potential advantages over live mycobacterial vaccines in terms of safety and quality control of the manufactured vaccine and are good candidates to improve the effect of bacille Calmette-Guérin. However, in order to confer the complex immunity required to protect against tuberculosis, it is possible that more than single antigens will be necessary. Progress to date with live attenuated *Mycobacterium tuberculosis* vaccines indicates that it is possible to design strains that are highly attenuated, even in immunodeficient animals. These classical vaccine candidates have to mimic natural infection as closely as possible without causing disease [38]. *Mycobacterium tuberculosis* mutant vaccine candidates have to induce long-term cellular immune responses, essential for effective protection against tuberculosis. New live vaccines should be stored lyophilised and current technology allows monitoring of any possible variations of genomic composition by comparative hybridisation experiments using DNA microarrays [10].

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