New Vaccine Against Tuberculosis: Current Developments and Future Challenges

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Tuberculosis (TB) continues to be a global health threat. BCG was developed as an attenuated live vaccine for tuberculosis control nearly a century ago. Despite being the most widely used vaccine in human history, BCG is not an ideal vaccine and has two major limitations: its poor efficacy against adult pulmonary TB and its disconcerting safety in immunocompromised individuals. A safer and more effective TB vaccine is urgently needed. This review article discusses current strategies to develop the next generation of TB vaccines to replace BCG. While some progresses have been made in the past decade, significant challenges lie ahead.

Key words: Tuberculosis, BCG, Vaccine

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*M. tb*), remains a major global health problem. TB, AIDS, and malaria are the 'big three' killer infectious diseases worldwide. TB causes ~2 million deaths annually and latently infects one-third of the world population (estimated 2 billion). Successful global TB control faces many obstacles including the difficulty of timely diagnosis, the lack of effective vaccines, and the fact that TB treatment requires many months of chemotherapy. The situation has been further complicated with the advent of *M. tb*/HIV co-infection and the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB. In 2006, the global burden of MDR-TB, defined as resistance to isoniazid and rifampin, was estimated at 500,000 cases [1]. In addition, the incident of XDR-TB, caused by MDR strains that are also resistant to a fluoroquinolone and at least one second-line injectable agent (amikacin, kanamycin, or capreomycin), is increasing in many countries [1]. A deadly association between HIV and TB has been known since the start of the HIV-epidemic. Of the 1.7 million people who died from TB in 2006, approximately 200,000 were co-infected with HIV [1]. In light of these developments, a new and effective vaccine is urgently needed, which is essential for reducing the estimated 8-10 million new TB infections that occur annually. According to the Global Plan to Stop TB (2006-2015), the introduction of effective TB vaccines will be an essential component of any strategy to control TB by 2050.

A brief history of BCG

Bacille Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis* (*M. bovis*), is currently the only available vaccine against TB. Around 1901, Albert Calmette and Camille Guérin isolated a virulent strain of *M. bovis* from the milk of a cow suffering from tuberculous mastitis. In order to minimize bacterial clumping and optimize animal infection experiments, Calmette added ox bile, a detergent, to the glycerol-soaked potato slices on which the *M. bovis* was cultured, which resulted in an isolate with unusual colony morphology and reduced virulence in guinea pigs. Recognizing the implications of these observations

in terms of vaccine development, Calmette and Guérin continued the serial *in vitro* passaging of this *M. bovis* strain for the next 13 years (1908-1921). During this time, experiments with diverse animal models, including guinea pigs, rabbits, dogs, cattle, horses, chickens and non-human primates, established both the safety and efficacy of BCG. When administered at different doses and by different routes, BCG was well tolerated and failed to produce tuberculous lesions. Moreover, BCG vaccination provided protection against a challenge with virulent strains. The first human trial occurred in July 1921. An infant was given three 2 mg doses (6 mg total; ~2.4×10^8 cfu) by the oral route [2]. There were no deleterious side effects and, most importantly, the child did not develop TB even though the infant's mother had died of TB shortly after giving birth. Over the next year, additional newborns were vaccinated and no ill effects were reported. For the first time, a safe and apparently effective vaccine was available for the prevention of human TB [2].

As early as 1924, cultures of BCG were distributed by the Institute Pasteur to laboratories around the world [3]. By 1926, at least 34 countries had received cultures from the Pasteur Institute. In 1927, another 26 countries received cultures of BCG [3]. The implementation of BCG varied from country to country, and several fascinating histories have been written about specific strains (e.g., BCG-Japan [4] and BCG-Moreau [5]). Because BCG is a live vaccine, it was necessary to transfer cultures to fresh media every few weeks. Despite efforts to standardize the growth and preparation of the vaccine, different passaging conditions were used. Although subculturing on potato or Sauton media was common, deep-culture methods were also used[6]. By the 1950s, numerous vaccine producers recognized the emergence of BCG substrains with distinct morphological, biochemical and immunological phenotypes [7-10]. Not until 1966, however, with the introduction of the "seed-lot system" as part of the '*Requirements for Dried BCG Vaccine*' initiated by WHO (WHO Expert Committee on Biological Standardization, 1966), was lyophilization of BCG strains started, and the process of *in vitro* evolution halted. As a result, dozens of distinct daughter strains emerged, including four that are currently in major use: BCG-Pasteur (1173P2), BCG-Japan (Tokyo-172), BCG-Danish (Copenhagen-1331), and BCG-Glaxo (1077).

Since 1974, BCG has been included in the WHO Expanded Program on Immunization [11]. More than 3 billion individuals have been immunized with BCG and >100 million doses of BCG are administered annually, making it the most widely used vaccine [11]. Clinical studies have confirmed that BCG protects children, providing >80% efficacy against severe forms of TB, including meningitis and miliary TB [12,13]. However, BCG has a limited effect against pulmonary TB in adults with variable efficacy estimates from clinical studies ranging from 0 to 80% [14]. Several hypotheses have been proposed to explain the variable efficacy, including differences in BCG strains used in clinical studies, differences in trial methods, differential exposure of trial populations to environmental mycobacteria, nutritional or genetic differences in human populations, and variations among clinical *M. tb* strains [15-20]. These explanations are not mutually exclusive and all may contribute to the heterogeneity in BCG efficacy.

It is now clear that BCG is not a single, isogenic strain, but instead comprises a number of substrains that exhibit phenotypic and biochemical differences [21]. During the past decade, comparative genome analyses of multiple BCG strains have uncovered extensive genotypic diversity, including both deletions and duplications, in BCG substrains [22-25]. A molecular phylogeny based on these studies has been established and is generally consistent with the historical records of BCG dissemination [22,26,27].

BCG strains also exhibit differences in residual virulence level [28-30]. However, side effects were often attributed to variations in the viability of vaccines during preparation procedures (e.g., freeze-drying) [21]. In other words, the observed differential virulence among BCG strains is often thought to have originated from quality control issues during manufacturing rather than reflecting true biological differences. Although differential virulence has been suspected to impact vaccine efficacy [7,31], definitive evidence, such as the direct implication of established virulence factors and/or virulence genes, has been lacking. Because of this, BCG strains are often considered to possess 'equivalent' vaccine properties and typically, only one BCG strain is selected for vaccine studies that compare the safety and efficacy of new vaccine candidates with "BCG". However, in the past decade, the advances in genomic techniques and knowledge of the virulence mechanisms of *M. tb* have offered unprecedented opportunities to re-evaluate these traditional assumptions. Indeed, our recent studies have provided direct evidence that the distribution of major mycobacterial virulence factors varies among BCG lineages [32,33], and suggests that different BCG substrains have different mechanisms of attenuation.

It is well recognized that BCG gives significant protection for only a limited period of time and is not effective in populations

already sensitized by mycobacterial antigens (e.g. by prior BCG vaccination, exposure to environmental mycobacteria, or latent TB infection) [12,13,34]. The period of immunity by BCG given at birth extends at best 10 to 20 years thus having little effect on the rate of TB in adults [35-37].

Current developments

Currently, the strategies for developing a new generation of TB vaccines are either to replace BCG with a stronger vaccine that provides a longer duration of protection or to design a vaccine used in conjunction with BCG that can be given at a later time point to boost existing immunity. Both of these approaches have advantages and disadvantages [38], and the vaccine candidates that have entered clinical trials include examples from both approaches.

The first example of the BCG replacement strategy is rBCG30, a recombinant BCG-Tice strain that overexpresses antigen 85B (Ag85B, Rv1886c) of *M. tb*, which is a major secreted protein and belongs to the mycolyl transferase family comprising Ag85A (Rv3804c), Ag85B and Ag85C (Rv0129c). Ag85A and Ag85B are highly immunogenic [39]. Guinea pigs immunized with rBCG30 and challenged with *M. tb* resulted in 0.5 to 1.0 logs fewer bacteria than animals immunized with BCG-Tice [40]. This was the first vaccine candidate that exhibited greater protection than BCG. Subsequent studies indicate that rBCG30 also appears to induce a longer duration of protection than BCG alone [41,42]. This vaccine candidate, developed by Marcus Horwitz's lab at UCLA, passed phase I clinical trials in 2004 [43]. A second vaccine candidate of this category is rBCG::Δ*ureC-llo*$^+$, which is a urease-deficient strain of BCG-Pasteur that expresses the listeriolysin O gene from *Listeria monocytogenes* [44]. Urease is deleted as a means of providing the optimal pH for listeriolysin function, which damages the phagosome membrane, allowing BCG leakage into the cytosol and increasing the amount of antigens avaliable for presentation to $CD8^+$ T cells [44]. BALB/c mice vaccinated with rBCG::Δ*ureC-llo*$^+$ showed a reduction in *M. tb* burden by ~1.0 log compared to mice immunized with the BCG control [44]. This vaccine has entered phase I clinical trials in 2008. Finally, others have attempted to make new vaccines by attenuating *M. tb*, reasoning that this would give the closest simulation of natural immunity occurring after *M. tb* infection. Examples include the *phoP* mutant of *M. tb* [45] and the non-replicating *M. tb* mutant strain (Δ*lysA* Δ*panCD*) that is auxotrophic for lysine and pantothenate [46].

The second strategy in TB vaccine development aims at boosting BCG-induced immunity, which includes administration of BCG as the "prime", followed by a "booster" inoculation with a subunit vaccine (DNA or protein) either to infants and young children before they are exposed to TB (i.e., boosting shortly after BCG vaccination) or as a separate booster to young adults (i.e., boosting several years after BCG vaccination) [38,47]. BCG is a live vaccine and the development of protective immunity after vaccination appears to require BCG replication in the host for a certain period of time [15,16]. However, this can be prevented by a pre-existing immune response that can cross-react to BCG, e.g., by exposure to environmental mycobacteria, prior BCG vaccination or *M. tb* infection [12,13,34]. As such, attempts to boost protection by giving multiple doses of BCG have proven ineffective [48]. Because of this, subunit vaccines are chosen as the booster and current research has focused on several antigens that induce strong INF-γ production [47].

Protection against TB requires a cell-mediated immune response, which is not fully understood but appears to involve multiple components including $CD4^+$ and $CD8^+$ T cells, unconventional T cells such as γδ T cells and CD1-restricted αβ T cells [49,50]. Currently, there is no proven immunological correlate of protection or "biomarker" for efficacy [51,52]. However, a critical role of INF-γ in the control of TB has been demonstrated in mice [53,54] and humans [55,56]. As such, INF-γ is currently used as a biomarker for TB vaccine selection, despite the fact that INF-γ alone is insufficient for protection against TB. The identification of *M. tb* antigens that induce strong INF-γ production has been the main strategy employed to uncover subunit vaccines. This was done by biochemical fractionation of *M. tb* protein mixtures, particularly the culture filtrate proteins [57-60]. Using this technique, several antigens were identified including small secreted proteins ESAT-6/Rv3875, CFP-10/Rv3874, TB9.8/Rv0287, and Mtb9.9A/Rv1793, the antigen 85 complex (Ag85A, B, C), and several PE/PPE family proteins (e.g., Rv1196/PPE18, Rv0195c/PPE14)[61-66]. Based on these studies, three fusion proteins Ag85B-ESAT-6, Ag85B-TB10.4 (Rv0288), and Mtb72f (Rv1196-Rv0125) were constructed and are presently the most advanced protein-based subunit vaccines. All three exhibit

protection similar to that obtained with BCG in mice and guinea pigs when formulated in selected Th1 inducing adjuvants [65,67,68]. Furthermore, Ag85B-ESAT-6 was protective in non-human primates [69] and Mtb72f was able to boost the protection afforded by BCG in cynomolgus monkeys [70]. All three recombinant protein vaccines have now completed phase I clinical trials and have either entered or are soon to enter phase II trials.

DNA based subunit vaccines have also been exploited, which uses viral vectors such as adenovirus or vaccinia virus for delivery to stimulate greater CD8 recognition of the expressed *M. tb* antigens. The first example of a DNA subunit vaccine is MAV-85A, a replication-deficient vaccinia virus expressing Ag85A. In rhesus monkeys, MAV-85A was shown to boost the protective efficacy of BCG [71]. This vaccine is now undergoing multiple phase I/II trials in Africa. Another example of a DNA subunit vaccine is Aeras-402, a replication-deficient recombinant adenovirus-35 expressing Ag85A, Ag85B, and TB10.4, which was shown to boost T cell responses in BCG-primed rhesus monkeys [72]. This vaccine is currently under phase I evaluation in South Africa.

Future challenges

While TB vaccine research has gained momentum in recent years, there are still major obstacles. A new generation of TB vaccines must offer greater protection than current BCG and be safe enough to be used in HIV-endemic countries. Currently, none of the subunit vaccines that are in clinical trials have exhibited greater efficacy than BCG in animal model studies, which is the reason they are considered a booster rather than a replacement for BCG. Although recombinant BCG strains (rBCG30, rBCG::Δ*ureC-llo*$^{+}$) and the attenuated *M. tb phoP* mutant consistently reduced the *M. tb* burden by ~1.0 log compared to BCG alone, it is not clear that whether this level of improvement is sufficient. In addition, the safety of these recombinant BCG and attenuated *M. tb* strains remains in question. In 2007, WHO revised its policy to recommend that BCG not be given to children known to be HIV-positive, even if asymptomatic, because of the substantially high risk of BCG-induced disseminated disease in HIV-infected individuals [73]. All clinical trials of new TB vaccines have so far excluded HIV positive individuals. While this cautious approach is logical, there is a need to evaluate new TB vaccines in HIV-infected populations because with an annual TB incidence rate of 5-10%, they are among the most in need of a new and effective vaccine. The *M. tb phoP* mutant would probably require further attenuation by additional mutations to ensure safety before entering clinical trials [71]. The non-replicating *M. tb* mutant strain (Δ*lysA* Δ*panCD*) is safer than BCG but does not afford the same level of protection as BCG [74]. Because of this, there is no guarantee that the leading candidates will progress through phase III clinical trials and registration. Experiences from HIV and malaria vaccine trials have taught us that it is important to continue preclinical research and to develop new and better vaccines for the pipeline. The search for new antigens [75,76] or combining different strategies (e.g., using the recombinant BCG strain that is urease-deficient and expressing perfingolysin O as the parental strain to overexpress Ag85B, coupled with subunit vaccines as booster [77]) to develop a better TB vaccine is on-going.

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