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REVIEW

Immunoprophylaxis of Tuberculosis: An Update of Emerging Trends

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Abstract Developing effective prophylactics to combat tuberculosis is currently in an exploratory stage. The HIV pandemic and emergence of multi- and extensively drug-resistant strains of *Mycobacterium tuberculosis* indicate that the current preventive measures against this ever-evolving pathogen are inadequate. The currently available vaccine BCG in its present form affords variable protection which usually wanes with aging. Various reasons have been cited to explain the discrepancies in the efficacy of BCG, including generic differences in the different BCG vaccine strains used in immunization program throughout the world. The low efficacy of BCG vaccine has promoted the search for novel vaccines for tuberculosis. The search strategies aim at completely replacing the existing vaccine and/or augmenting/improving the current BCG vaccine. Among new vaccine candidates are live attenuated *M. tuberculosis* vaccines, recombinant BCG, DNA vaccines, subunit vaccine, and fusion protein-based vaccines. More than 200 new vaccine candidates have been developed as a result of research work over the past few years. To date, at least eight vaccine candidates are undergoing clinical evaluation, with a few of them successfully qualifying in the first phase of clinical testing. These recent advances present an optimistic insight whereby a new tuberculosis vaccine might be expected to be available for public use in the next few years.

Keywords BCG · *M. tuberculosis* · Vaccine · Prophylactics

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The mycobacterial diseases leprosy and tuberculosis (TB) have inflicted mankind since time immemorial. While leprosy has shown a decline over the past years, TB continues to cause death and suffering. Despite the fact that TB is a disease for which the causative organism was discovered as early as in 1882 and for which a vaccine was developed in 1921, the disease has expanded into greater dimensions. With eight million new cases and more than 1.5 million deaths each year (World Health Organization 2007), TB as a disease has attracted the attention of both the developing and the developed world. The HIV-AIDS synergy and the emergence of multi- and extensive drug-resistant (MDR and XDR) strains of *Mycobacterium tuberculosis* have extended the frightening dimensions to the current scenario. This evokes a general consensus that in addition to the currently available tools, directly observed therapy, short course (DOTS) and modern diagnostic tests, new chemo- and immuno-prophylactics are urgently warranted to combat the disease.

The DOTS program adopted by the World Health Organization (WHO), though highly effective in reducing prevalence in the targeted regions, is expensive and requires active partnership of the public health infrastructure and administration for optimal success. While effective as a therapeutic measure, it does not protect susceptible people from those infected. It is here where

vaccines come to the fore by helping to shield the susceptible population from disease. An attractive feature of vaccine is the potential for a highly beneficial affect with relatively low costs of production and distribution to large populations. In the long run, vaccination will be far more cost effective than chemotherapy because the great majority of patients with active TB belong to low-income groups of mainly developing countries. The currently available vaccine, BCG, has a long safety profile with a global immunization coverage of more than any other vaccine.

The Origin of BCG Vaccine

In 1908, the French scientists Calmette and Guerin isolated *Mycobacterium bovis* from a cow with tuberculous mastitis. They cultured this isolate in a glycerinated beef/bile/potato medium, sub-culturing every 3 weeks until, 13 years and 230 subcultures later, the resultant strain (Calmette et al. 1921) showed no pathogenicity to guinea pigs or cattle. In 1921, the newly developed vaccine was administered to infants in France, where it showed remarkable success, reducing the mortality rate to as low as 90%. The resulting vaccine was believed to have acquired a balance between reduced virulence and preserved immunogenicity (Calmette and Plotz 1929). From 1921, when BCG began to be used for human immunization, to 1961, when the WHO recommended lyophilization and storage at −80°C for vaccine cultures, BCG had already undergone passage for 40 years to maintain viability. Meanwhile, its distribution to different laboratories around the world had resulted in the emergence of a number of daughter strains, which are currently known by their respective geographical names. These daughter strains/substrains are different not only from each other, but also from their parent.

BCG Vaccine Trials

The level of protection afforded by BCG has varied considerably in the numerous vaccine trials conducted throughout the world. Large-scale studies to test the efficacy of BCG have been conducted since the 1930s. A 60 year follow-up study in American Indians and Alaska natives showed that the efficacy of BCG vaccine persists for 50–60 years (Aronson et al. 2004), roughly amounting to lifelong protection. However, trials in countries with a high incidence of TB showed a lower degree of protection by BCG (Bloom and Fine 1994). The last major vaccine trial involving hundreds of thousands of people conducted in an TB endemic country like India, popularly known as the Chingleput trial, showed that BCG had no protective efficacy against TB in this community (Tuberculosis Prevention Trial 1979).

Various hypotheses have been put forward to explain these discrepancies in BCG protective efficacy. Researchers believe that differences in the geography, operation, and demography of the trials along with different study designs were responsible for the variability in protective efficacy. Factors which were held responsible are: inappropriate storage of the vaccine, doses and immunization schedules (BCG only at birth, BCG once in childhood or repeated/booster BCG) (Fine et al. 1999), biological variability due to strain-specific mutation and the immunogenic character of the vaccine (Aguirre-Blanco et al. 2007; Brosch et al. 2007; Leung et al. 2008; Liu et al. 2009), the genetic variability among, and different ages of, the vaccinated individuals, latent infection by *M. tuberculosis* in the vaccinated population, immunological cross-reactivity between BCG and environmental mycobacterial strains prevalent in different parts of the world (Brandt et al. 2002), and the strain of *M. tuberculosis* inflicting the target population.

The protective effect of BCG against pulmonary disease is only 50% (Brewer 2000; Colditz et al. 1994). Despite the doubts raised and efforts to improve/replace the current vaccine, medical practitioners are largely agreed that BCG should continue to be administered at birth (as is being done throughout the developing world), where it confers at least a consistent and reliable protection of up to 80% against severe and disseminated disease, particularly tuberculous meningitis and miliary TB in the first 10 years of life (Trunz et al. 2006; Walker et al. 2006).

BCG, a Plural Term: Efficacy

Every year, more than 120 million doses of BCG are being supplied by the WHO to more than 100 countries of the world. Worldwide, the most commonly used strains are currently BCG-Denmark, BCG-Japan, and BCG-Bulgaria, since these strains are supplied by the UNICEF itself. Many developing countries use several BCG vaccine strains concomitantly. In contrast, one strain is usually licensed for use in most developed countries (Ritz et al. 2008). In India, a BCG vaccine laboratory was established in 1948 to cater to the indigenous vaccine need as well as to supply some neighboring countries. Since 1961, Danish strain 1331 is being used here for vaccine production. Most countries import BCG vaccine from one of the international manufacturers, but few countries produce their own vaccine. Examples of nationally produced BCG vaccines are BCG-Poland based on BCG-Moreau, BCG-Romania based on BCG-Pasteur, and BCG-China based on BCG-Denmark (Ritz et al. 2008).

Comparative genomic studies of different strains of BCG using microarray technology have shown that BCG vaccines have lost important genes during the laboratory attenuation process from the parent strain. Important proteins present in the parental strain are either absent (ESAT-6, and CFP-10, MPT64) or not expressed (MBP70 and MBP83) in several daughter strains (Behr 2002). Based on the regions of difference (RD) and SNPs, BCG vaccines have been divided into the early strains represented by BCG-Japan, -Birkhaug, -Sweden, and -Russia, and late strains, including BCG-Pasteur, -Denmark, -Glaxo, and -Prague (Fine et al. 1999). Brosch et al. performed comparative in vitro transcriptomic analysis across the early and late BCGs and suggested that the early BCG vaccines may confer better protection against TB than the late ones (Brosch et al. 2007).

Immunological studies in both animals and humans have also shown strain-specific protection variations in BCG. The BCG-Pasteur, -Denmark, and -Glaxo strains showed better protection in mice, while BCG-Denmark proved to be an effective strain in guinea pigs. In a study by Castillo-Rodal et al., mice were immunized by ten different BCG vaccines and after a few weeks were challenged with intratracheal *M. tuberculosis* H37Rv (Castillo-Rodal et al. 2006). When lung pathology was measured after 2 and 4 months, each strain showed a specific cytokine profile. Other animal studies have also shown different results (Collins 1985; Lagranderie et al. 1996).

Human studies on BCG vaccination have yielded varying results depending on the study design, immunological parameter, and strain investigated (Aronson et al. 2004; Davids et al. 2006; Gorak-Stolinska et al. 2006; Hussey et al. 2002). A recent study by Aguirre-Blanco et al. showed that the commonly used BCG vaccines, namely the Danish, Glaxo, and Pasteur, elicit different in vitro magnitudes of T-cell activation and interferon (IFN)-γ in healthy BCG-vaccinated individuals (Aguirre-Blanco et al. 2007). In another study (Wu et al. 2007), the gene expression patterns of 107 children immunized with different BCG strains were studied. One year after immunization, PBMCs were stimulated with *M. tuberculosis* antigens and the expressions of cytokine genes were investigated. BCG-Denmark- and BCG-Brazil-immunized children expressed higher levels of cytokines responsible for the adaptive immune response, while those immunized with BCG-Japan exhibited a higher pro-inflammatory cytokine expression. The group proposed that different strains of BCG could activate different immune pathways, which may affect long-term vaccine efficacy.

Towards a New Vaccine

Although it would be premature at this stage to decide which strains should be included in or excluded from the current vaccination programs, it can be said with precision that some vaccine strains definitely perform better than others. Although the efficacy of BCG as a singular vaccine or BCG as a plural is being explored by a section of the scientific community, another section insists on improving the current BCG itself or totally replacing it. Current attempts to develop better immuno-prophylactics for TB are based on two approaches: replacing or boosting BCG. The former strategy insists on replacing BCG with a more effective vaccine obtained either through gene-deletion mutants of *M. tuberculosis* or by reintroducing important antigenic factors into the existing BCG vaccine strains. Viable attenuated mycobacterial strains, when used as vaccines, will present a spectrum of antigens and will obviously sensitize a broad variety of the host T-cell population. The other strategy is based on developing a booster vaccine to prolong adult immunity after childhood immunization with BCG, which is a regular practice in TB endemic countries. Subunit and fusion-protein vaccines are the best candidates for booster vaccination. With the aid of modern techniques, several research groups have developed more than 200 new vaccine candidates. They include live attenuated *M. tuberculosis* vaccines, reengineered BCG and vaccinia-vectored vaccines, DNA vaccines, and subunit vaccines and fusion proteins, combined with novel adjuvants and delivery systems. The critical issues in the clinical trials of the different classes of vaccines will be different for one vaccine class to another. For live vaccines (e.g. recombinant BCG strains, rationally attenuated *M. tuberculosis*) the principal concern is safety, for the subunit vaccines (e.g. proteins or peptides) safe and effective adjuvant development is the challenge, and for DNA vaccines the issue is effective delivery systems which can ensure long-lasting immunity.

Live Mycobacterial Vaccines

Many alternative living and non-living mycobacterial members have been explored as putative TB vaccines. Live vaccines include less virulent, naturally attenuated mycobacteria or attenuated mycobacteria such as auxotrophic *M. tuberculosis*. Naturally attenuated strains of mycobacteria, such as *M. vaccae*, *M. microti*, and *M. habana*, can serve as potential vaccines for TB. Live *M. vaccae* and *M. microti* have been suggested as vaccine candidates, but animal experiments showed variability in their protective efficacy (De Bruyn and Garner 2003). Recombinant *M. vaccae* and *M. smegmatis* (Abou-Zeid et al. 1997; Orme 1997) that express *M. tuberculosis* epitopes have also been reported. However, none of them performed better than BCG in the experimental models. Live auxotrophic *M. tuberculosis* strains are interesting vaccine candidates,

but their major drawback is the difficulty in achieving an appropriate level of attenuation without compromising their immunogenicity.

Knocking out certain genes or inducing one or more mutations in an essential metabolic pathway generates auxotrophic mutants. Auxotrophic mutants of *M. tuberculosis* have been generated to produce improved live vaccines for TB (Hernandez Pando et al. 2006; Sambandamurthy and Jacobs 2005; Smith et al. 2001), but only a few of these mutants conferred better protection than the standard BCG in animal models. Mice immunized with *M. tuberculosis* purine (Δpur C), leucine (Δleu D), or lysine (ΔlysA) auxotrophic strains did not show protection comparable to that conferred by BCG (Hondalus et al. 2000; Jackson et al. 1999; Pavelka et al. 2003). Some *M. tuberculosis* mutants have afforded a level of protection comparable to or even greater than that of BCG in animal models. Auxotrophic mutants of *M. tuberculosis* for cysteine and methionine (ΔcysH) and for glutamine (ΔglnA1EA2 and ΔglnA1) were found to afford an equivalent protection to BCG against an aerogenic challenge of *M. tuberculosis* in C57BL/6 mice (Lee et al. 2006; Senaratne et al. 2007). The *M. tuberculosis* phoP mutant strain SO2 was shown to have a reduced multiplication capability in mouse macrophages and in vivo using the mouse intravenous infection model (Perez et al. 2001). Martin et al. showed that the phoP mutant strain SO2 in BALB/c mice conferred an equivalent protection to BCG, while in guinea pig experimental models the level of protection was found to be superior to that of BCG vaccination, as measured by guinea pig survival and reduction in disease severity in the lung (Martin et al. 2006). Aguilar et al. also compared the immunological responses and protective efficacy of the *M. tuberculosis* SO2 strain and found a similar level of protection to BCG in BALB/c mice (Aguilar et al. 2006). Similarly, pantothenate auxotroph (Sambandamurthy et al. 2002), double lysine and pantothenate auxotrophs (Sambandamurthy et al. 2005), and double leucine and pantothenate auxotrophs (Sampson et al. 2004) were found to be fully attenuated in an SCID mouse model and protective in mice and guinea pig models. *M. tuberculosis* region of difference RD1-deleted mutants (H37Rv: ΔRD1) and Rv 3763 gene-deleted mutants (encoding a 19 kDa lipoprotein) have also been claimed suitable vaccine candidates for their vaccinogenic character (Henao-Tamayo et al. 2007; Sherman et al. 2004). The *M. tuberculosis* fadD26 mutant, with impaired phthiocerol dimycocerosates synthesis, and *M. tuberculosis* mce-2- and mce-3- (mammalian cell entry genes) deleted mutants conferred better protection than BCG, as evident by less tissue damage (pneumonia) and lower colony-forming units in BALB/c mice (Aguilar et al. 2006; Infante et al. 2005). In the wrath of the growing HIV pandemic, a double-deletion mutant of *M. tuberculosis* was developed by deleting the RD1 region (ΔRD1) and two genes required for pantothenate synthesis (ΔpanCD) by Sambandamurthy et al. which was proposed to be considered as a human vaccine candidate for protecting both healthy and HIV-infected individuals against TB (Sambandamurthy et al. 2006). There is an urgent need for their evaluation in other animal models, including non-human primates.

Recently, Gupta et al. studied the protective potential of "*Mycobacterium* w" in both live and killed forms through the parenteral and aerosol routes (Gupta et al. 2009). They reported that Mw activates macrophage activity as well as lymphocytes. Mw immunization by both the parenteral route and aerosol administration gave higher protection than BCG given by the parenteral route in the mouse model of TB.

DNA Vaccines

DNA vaccines containing plasmids encoding important mycobacterial antigens such as members of the mycolyltransferase family Ag85 complex (Baldwin et al. 1998; Kamath et al. 1999; Ulmer et al. 1997), the phosphate-binding protein family Pst-3 (Tanghe et al. 1999), the 38 kDa protein (Fonseca et al. 2001), ESAT-6 (Brandt et al. 2000), and heat-shock proteins 60, 65, and 70 (Ferraz et al. 2004; Johansen et al. 2003) have been extensively explored in laboratory animal models. Recently, Romano et al. reported the vaccine potential of an *M. tuberculosis* antigen of the PPE protein family, PPE44 (Rv2770c) (Romano et al. 2008). Vaccination of mice with a plasmid DNA vaccine coding for PPE44 or recombinant PPE44 protein formulated in adjuvant generated protection equivalent to that of BCG vaccination.

Some researchers claim that plasmids coding for more than one antigen are more effective than those coding for a single antigen. In two separate studies by Tian et al. a divalent DNA vaccine encoding Ag85B and MPT64 (Tian et al. 2004) and MPT83 and MPT64 (Tian et al. 2005) was found to impart better immunoprophylaxis than that of either monovalent DNA vaccine or of BCG itself in vaccinated mice. On the other hand, when Chang-hong et al. used a divalent DNA vaccine encoding Ag85B-ESAT6 they found that the divalent vaccine confers protection equal to BCG (Chang-hong et al. 2008).

DNA vaccines offer a twin advantage. The first is that due to their injection into the muscle mass and presentation by dendritic macrophages (present in the muscle mass), strong TH-1 as well as CD8+ T-cell mediated cytotoxic responses are provoked, which is important to combat *M. tuberculosis* infection. The other advantage of DNA

vaccines is that they can be constructed together with immunostimulatory DNA sequences such as the CpG sequences (Iwasaki et al. 1997; Tokunaga et al. 1999) and cytokines to enhance their immunogenicity. Interleukins were found to possess promising adjunctive modality and the capability to enhance immunogenicity of many DNA vaccines, such as DNA vaccines encoding antigen 85B with interleukin (IL)-23 (Wozniak et al. 2006), antigen 85A with IL-21 (Dou et al. 2008), heat-shock protein 65 (HSP65) with IL-12 (Yoshida et al. 2006), and HSP65 with IL-2 (Wang et al. 2008). Xu et al. reported the adjuvant character of Flt3 ligand (FL), a growth factor of dendritic cells (Xu et al. 2008). A recombinant DNA vaccine encoding ESAT-6 and FL when injected into mice was found to effectively promote a T cell-mediated immune response.

In spite of some initial discouraging experimental findings (Taylor et al. 2003), DNA vaccines have generally proved safe in preclinical and clinical studies (Schalk et al. 2006). DNA vaccines exhibit no signs of autoimmunity, development of anti-nuclear or double-stranded DNA antibodies, or plasmid DNA integration into chromosomes (Cebere et al. 2006; Coelho-Castelo et al. 2006; Moorthy et al. 2003). Recent approaches to improving the performance of DNA vaccines involve the use of delivery systems and adjuvants (Greenland and Letvin 2007; Jechlinger 2006). One such delivery system is the gene-gun technology. Tanghe et al. compared the protective efficacy of Ag85A DNA vaccine on C57BL/6 mice through intramuscular injection versus the gene-gun delivery method and inferred that the route of administration of DNA vaccine plays a profound role on its efficacy (Tanghe et al. 2000). Among other important delivery methods for DNA vaccine are a cationic lipid formulation which was found to improve the immunogenecity of Ag85B vaccine (D'Souza et al. 2002), spermidine-polyglucin conjugate encapsulating the ESAT-6 antigen (Nosareva et al. 2008) and cationic microparticles (Cai et al. 2005; Denis-Mize et al. 2003). An alternative delivery system based on a genetically modified mutant of the non-pathogenic commensal bacterium *Escherichia coli* was developed and used to deliver DNA vaccines against two *M. tuberculosis* proteins, FbpA and HtpX, following intranasal administration. Both DNA vaccines were able to induce an antigen-specific T-cell response, while the DNA vaccine encoding HtpX provided significant protection to mice against *M. tuberculosis* challenge (Brun et al. 2008).

Yoshida et al. compared the protective efficacy of a DNA vaccine combination expressing mycobacterial HSP65 and IL-12 when used for immunizing mice with either gene-gun bombardment or the hemagglutinating virus of Japan (HVJ)-liposome method (Yoshida et al. 2006). In a mouse model, a single gene-gun vaccination with the combination of HSP65 DNA and mIL-12 DNA provided remarkably high protection against challenge with virulent *M. tuberculosis*; bacterial numbers were 100-fold lower in the lungs of mice vaccinated with HSP65 + mIL-12 compared with BCG-vaccinated mice. To explore the clinical use of DNA vaccine further, they evaluated HVJ-liposome-encapsulated HSP65 DNA and mIL-12DNA (HSP65 + mIL-12/HVJ). The HVJ-liposome method improved the protective efficacy of HSP65 DNA vaccine compared with gene-gun vaccination as evidenced by the emergence of IFN-γ-secreting T cells and activation of proliferative T cells and cytokines (IFN-γ and IL-2). The protection offered by the HSP65 + IL-12/HVJ DNA vaccine was superior to that of BCG in the mice models (Yoshida et al. 2006). Later, Okada et al. inferred that this DNA construct harbored both therapeutic and protective efficacy, as evident from CD8+ and CD4+ T-cell counts in infected mice and on the basis of the number of colony-forming units of MDR-TB and the survival of XDR-TB-challenged mice (Okada et al. 2009). Further, when tested on cynomolgus monkey (a macaque), which is currently the best animal model of human TB, this DNA vaccine could bestow therapeutic efficacy (survival and immune responses) upon them as well, supporting its claim to be considered for clinical trials.

Subunit and Fusion Protein-based Vaccines

Subunit vaccines containing one or more pure or semi-pure antigens are based on the assumption that a few antigens are sufficient to induce and maintain a protective immune response. Besides being stable and safe even in immunocompromised individuals, the possibility to include only the useful components and exclude the detrimental ones (present in live attenuated mycobacterial vaccines) is an additional advantage. In the development of subunit vaccines, the major focus has been on vaccines based on culture filtrates or secreted proteins purified from culture filtrates of *M. tuberculosis*, such as Ag85 complex and ESAT6 and intracellular proteins such as Mtb32/Mtb39 fusion protein Mtb 72F. *M. tuberculosis* Ag85 complex consists of three closely related mycolyl transferases of 30–33 kDa molecular weight, i.e. Ag85A–C. Ag85A and Ag85B have been reported to be ideal vaccine candidates in a number of studies involving both DNA and protein vaccines (Lozes et al. 1997; Mustafa et al. 2000; Yadav and Khuller 2001). Fusion proteins based on Ag85B and ESAT-6 administered with a strong adjuvant provided protective immunity comparable to BCG-induced protection over a broad dose range (Weinsrich Olsen et al. 2001).The 6 kDa early secreted antigen target molecular ESAT-6, an immunodominant antigen, has also been a

leading vaccine candidate for DNA and protein vaccines (Brandt et al. 2000).

A fusion molecule comprising the immunodominant secreted proteins of *M. tuberculosis* Ag85B-ESAT-6 has proven highly efficient in animal models, ranging from mice to primates, and better than the single antigens (Doherty et al. 2004; Langermans et al. 2005; Olsen et al. 2004; Weinrich Olsen et al. 2001). Interestingly, since ESAT-6 is currently also being used as a diagnostic tool to detect cell-mediated immunity signaling ongoing infections, efforts to replace ESAT-6 in the fusion molecules led to the discovery of TB10.4, a member of the ESAT family but also present in BCG (Dietrich et al. 2005; Skjot et al. 2002).

The subunit vaccine Mtb72F codes for a 72 kDa polyprotein, Mtb32(C)-Mtb39–Mtb32(N). Immunization of mice with Mtb72F protein in the adjuvant AS01B elicited strong IFN-γ and antibody responses for all the three components of the vaccine and a strong CD8+ response directed to the Mtb32(C) epitope. Mtb72F immunization to BALB/c mice and guinea pigs protected them against aerosolic infection with virulent *M. tuberculosis*. Mtb72F has shown good protection against TB in animal models ranging from mice (Skjot et al. 2000), guinea pigs (Brandt et al. 2004), rabbits (Tsenova et al. 2003), and macaques (Reed and Lobet 2005). The available experimental data paved the way for these two fusion polyprotein vaccines toward clinical trials.

The search for novel immunodominant antigens of *M. tuberculosis* has nevertheless continued and new immunodominant genes for subunit vaccination are being unraveled (Bertholet et al. 2008). Sable et al. suggested the potential of multicomponent subunit vaccination against TB based on strongly immunogenic proteins of *M. tuberculosis* (Sable et al. 2005). They evaluated the inherent immunogenicity of 102 polypeptides purified from the low-molecular-weight region below 40 kDa in a mouse model of immunization and, based on the high degree of immunogenicity, constructed a multicomponent subunit vaccine consisting of five immunodominant polypeptides (CFP-25, CFP-20.5, Ag85B, AG85A, and CFP-32). When administered with DDA-MPL adjuvant in C57BL/6 J mice, the polyprotein vaccine conferred protection comparable to BCG and total mycobacterial culture filtrate protein-based vaccines. Fusion molecules based on ESAT-6 and MPT64 (Bai et al. 2008), PPE44 (Romano et al. 2008), and HSP65 and human IL-2 (Shi et al. 2009) have also been proposed as alternative vaccine candidates for *M. tuberculosis* infection. Identification and appropriation of novel adjuvant systems for subunit vaccines is as important as discovering immunodominant antigens to appropriate, selectively induce, protective T-cell responses. Adjuvants that were found suitable for use with subunit vaccines and are in or approaching clinical trials are LTK63, Lipovac, AS2, IC31, Montamide, ISCOM, and OM-174 (Doherty and Andersen 2005). Recombinant modified vaccinia virus Ankara expressing Ag85A (MVA85A) used as a booster after primary immunization with BCG became the first subunit vaccine for tuberculosis in clinical trials which have now made their way to phase II (McShane et al. 2004). Several of these fusion polyproteins, such as Ag85B-ESAT-6 (H1), Ag85B-TB10.4 (H4), and MTB72f (by fusion of MTB32 and MTB39) (Agger et al. 2006; Dietrich et al. 2005; Skeiky et al. 2004), have already qualified for phase I of clinical trials. The success with subunit vaccines is evident from the fact that of more than 200 vaccines selected/screened among potential TB vaccine candidates, a large number of them are based on subunit and fusion proteins.

Reengineered BCG/Recombinant BCG

BCG lost a number of genes before acquiring its present form during the long-term in vitro laboratory attenuation process. Reintroducing and re-expressing some of the genes encoding the immunodominant antigens or immunostimulatory genes in BCG that were deleted can improve the efficacy of BCG. This strategy could be useful in exploiting the long safety record associated with BCG and economic constraints in mass manufacture. Recombinant BCG expressing various cytokines such as IL-2, IFN-γ, or granulocyte macrophage colony-stimulating factor have been shown to improve the response against *M. tuberculosis* antigens (Murray et al. 1996; Wangoo et al. 2000), and recombinant BCG expressing listeriolysin of *Listeria monocytogenes* showed an enhanced capacity to stimulate CD8+ T cells (Hess et al. 1998). Recently, a recombinant BCG expressing Ag85B and the PPE protein Rv3425 from *M. tuberculosis* (rBCG: Ag85B–Rv3425) has been advocated as a strong vaccine candidate (Wang et al. 2009). rBCG30 is a recombinant BCG vaccine in which Ag85B is overexpressed. This 30 kDa enzyme, which is involved in outer cell-wall synthesis, is a key component in several TB vaccines and has already proved good in phase I clinical trials (Horwitz and Harth 2003). Another recombinant BCG vaccine which qualified for phase I clinical trials which commenced in the end of 2007 is rBCGΔureC:Hly, which is reengineered to express all the antigens expressed by standard BCG along with listeriolysin (hly), which can perforate the phagosome membrane. The gene (ΔureC) encoding the urease enzyme was additionally deleted to avoid neutralizing the phagosome, as this would reduce the activity of Hly (Grode et al. 2005).

Final Considerations

Research to develop improved TB vaccines seems to have reached a decisive point. More than 200 vaccine candidates have been proposed as a result of work over recent years in laboratory experimental models and a few of them are undergoing clinical testing. The vaccine candidates undergoing clinical testing include candidates from both the BCG replacement and BCG booster groups. BCG replacement vaccines undergoing clinical testing are rBCG30 (recombinant BCG overexpressing Ag85B) and rBCG ΔureC:Hly (recombinant BCG gene deletion, ΔureC deleted:listeriolysin expressed) (Grode et al. 2005; Horwitz and Harth 2003). Vaccines designed to serve as boosters for previous BCG immunization are Hybrid1, which is an Ag85B-ESAT-6 fusion protein in IC31 adjuvant given intradermally or in LTK63 adjuvant for mucosal delivery (Agger et al. 2006; Dietrich et al. 2006), Hy Vac4, which is an Ag85B-TB10.4 fusion protein in IC31 adjuvant for intradermal injection (Dietrich et al. 2005), Mtb72F, a fusion protein of MTB32 and MTB39 in ASO2A adjuvant for intrademal injection (Skeiky et al. 2004), and MVA85A, recombinant live or non-replicating vaccinia virus Ankara expressing Ag85A for intradermal injection (McShane et al. 2004). Another vaccine to boost previously induced BCG immunity in HIV-positive individuals is based on a killed whole *M. vaccae* suspension and is administered intradermally (Waddell et al. 2000).

From the viewpoint of a policymaker or health worker, the fact that TB is a disease afflicting mainly the low-income strata of society is sufficient reason to explore the vaccine world; on the other hand, for a private profit-making biotechnology or pharmaceutical company this makes vaccine research a venture involving greater financial risks than returns. Nevertheless, the active participation of several governmental and nonprofit organizations, novel tuberculosis vaccine development, as well as improvement in the currently existing BCG vaccine have continued. In recent years, with increasing aid from public funds such as the European Union, the National Institutes of Health, the AERAS Global TB Vaccine Foundation, the Bill and Melinda Gates Foundation, and more, TB vaccine research has gained great momentum. Although most of the work is currently being carried out by public and public–private partnerships, it is hoped that the commercial value associated with a novel TB vaccine will attract a larger investment from the private sector in the near future. This is desirable because to be able to transit from laboratory studies to clinical trials during the course of vaccine development, many strategic technical implications have to be applied. Phase I clinical trials are relatively small and inexpensive and are often conducted by the vaccine developer with conventional research funds, while phase III trials are large and costly endeavors requiring the manufacture large batches of vaccine under Good Laboratory Practices/Good Manufacturing Practices conditions, reexamination and repetition of the biological characteristics of the developed product in animal models, selecting field trial sites, and maintaining a cohort with regulatory authorities and funding sources (Sadoff and Hone 2005).

All TB vaccine candidates currently under examination are designed as pre-exposure vaccines intended to stimulate the host immune response to combat a future infection with *M. tuberculosis*. But what about the masses of those who are already infected or were infected once in their life with the disease? Post-exposure vaccines, which induce protective immunity in already infected individuals by strengthening the immune response elicited by natural infection with *M. tuberculosis*, should also be explored by vaccine researchers. Nevertheless, with improved knowledge of *M. tuberculosis'* pathogenic factors and the host protective immune response, this task will not prove to be impossible.

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