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Review Article

Development of Vaccination Strategies: From BCG to New Vaccine Candidates

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ABSTRACT

During recent years, extensive development has been made to improving vaccines for tuberculosis. This is due to the presence of genome sequences of diverse mycobacterial species and *Mycobacteroum tuberculosis* (*M. tuberculosis*) isolates which has led to advances in the characterization of genes and antigens of *M. tb* and better realization of protective immune responses to the disease in both animals and humans. This review summarizes vaccine types, reasons for variable efficacy of BCG, latest advances in tuberculosis vaccine development and major vaccine design strategies.

Keywords: Tuberculosis, vaccines, bacille Calmette-Guerin (BCG), Region of difference (RD)

INTRODUCTION

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB). The proof for the presence of tuberculosis in ancient times was found when fragments of the spinal column from Egyptian mummies from 2400 B.C. showed definite signs of tuberculosis (Morens, 2002).

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Tuberculosis ranks second only to human immunodeficiency virus as a cause of death from an infectious agent, with 1.7 million deaths from TB in 2007 (Philippe *et al.*, 2009). The most important factor for TB prevalence is the incidence of HIV (Simonney *et al.*, 2007). MDR-TB is another incidence factor for TB disease, as TB bacilli resistant to at least isoniazid and rifampicin, the two most powerful anti-TB drugs (von der Lippe *et al.*, 2006).

Calmette and Camille Guerin is the current vaccine used against TB. BCG has been administered worldwide. BCG offers unique advantages as a vaccine because:

(1) it is unaffected by maternal antibodies and can therefore be given at any time after birth; (2) BCG is usually given as a single dose eliciting a long-lasting immunity; (3) it is stable and safe; (4) BCG can be administrated orally; and (5) it is inexpensive to produce compared to other live vaccines. In addition, the extraordinary adjuvant properties of mycobacteria make them an attractive vector for the development of recombinant vaccines (Reginaldo *et al*., 2009). However, doubts about its efficacy are increasing, and these have been particularly reflected by its highly variable protective efficacy in controlled clinical trials. A number of suggested reasons for the variation of BCG efficacy include the genetic variability amongst, and different age of, the vaccinated individuals and immunological cross reactivity between BCG and environmental mycobacterial strains prevalent in the different parts of the world (Brandt *et al.*, 2002).

Recently, there has been growing interest in improving vaccine as an alternative to BCG for controlling TB. Many vaccines have been presented, and they can be classified mainly into live-attenuated vaccine (Kamath *et al.*, 2005), subunit vaccine (such as Ag85 and ESAT-6) (Brooks *et al.*, 2001; Orme, 2006; Olsen *et al.*, 2001) and DNA vaccines (Nor & Musa, 2004; Kaerch *et al.*, 2002). Highlighting BCG defects and finding the correct strategies for vaccine design may possibly help to produce effective vaccines against tuberculosis.

TUBERCULOSIS VACCINES

Live Attenuated Vaccine

An effective cell mediated immunity (CMI) response is important in controlling tuberculosis because it is an intracellular pathogen. The fact that live-attenuated strains are generally more potent than non-living vaccines in stimulating CMI responses supports the use of live vaccines. Moreover, the immune responses elicited following vaccination highly resemble the responses that follow a natural infection, since most of the antigens are expressed *in vivo* (Sambandamurthy & Jacobs, 2005).

In spite of the fact that live vaccines are effective against intracellular pathogens, the development of these vaccines is difficult because of the difficulty in balancing between producing required level of attenuation and immunogenicity. The strength of the immune response is a function of the amount of antigen expressed and T-cell response against individual antigens. Obviously, the high attenuation of BCG strain for several decades of years has led to limited replication *in vivo* and subsequently to BCG failure (Sambandamurthy & Jacobs, 2005).

The first strategy of researchers, i.e. improving live-attenuated vaccine for tuberculosis, has focused on strengthening the immunogenicity of vaccine or producing genetically modified recombinant BCG strains by insertion or over-expression of immunodominant antigens or immunostimulatory cytokines of *M. tuberculosis*. BCG30 was the first recombinant BCG vaccine reported to induce higher protective immunity to TB than the standard BCG vaccine in animal models. This vaccine which is constructed by a plasmid expresses the 30 kDa major secreted proteins. Another example is Ag85B which is introduced into BCG Tice strain. The plasmid containing Ag85B gene has been shown to be stable for long periods of time without antibiotic selective pressure *in vitro* and *in vivo* (Kamath *et al.*, 2005; Sambandamurthy & Jacobs, 2005).

Extending the idea of inducing BCG vaccine to express additional *M. tuberculosis* antigens, more than one *M. tuberculosis* antigen was introduced to BCG Pasteur strain by introducing RD-1 (region of difference) locus of *M. tuberculosis*, which expresses immunoprotective proteins such as ESAT-6 and CFP-10. In spite of the capability of ESAT-6- to stimulate specific $CD4^+$ T cells following immunization, this vaccine candidate displayed increased virulence in the animal model compared to the parental strain (Kamath *et al.*, 2005; Sambandamurthy *et al.*, 2005).

To improve the immunogenicity of BCG vaccine, recombinant BCG has been modified to produce (rBCGΔUreC: Hly) vaccine through deletion and addition of genes into BCG pasture strain. The deletion was done for urease gene, which normally enables mycobacteria to block the acidification of the early phagosome of the macrophage. As a result, urease deletion can prevent neutralization of the acidic pH in phagosomes. The vaccine (rBCGΔUreC: Hly) has also been reengineered by over-expression of listeriolysin O, which increases MHC class I presentation and $CD8^+$ T-cell responses. This recombinant BCG (rBCGΔUreC: Hly) was found to be safe for mice and provide protection against aerosol TB in the mouse model (Kamath *et al.*, 2005; Tony *et al.,* 2011).

The second strategy for producing tuberculosis vaccine is by using attenuated mycobacteria such as auxotrophic *M. tb* strains like *Pho*P mutant of *M. tuberculosis*. This vaccine was produced by disrupting the gene of *phoP* in the MT103 strain of *M. tuberculosis*. *phoP* importance belongs to its involvement in regulating a number of genes in *M. tuberculosis*. PhoP has also been identified to be a virulence factor, as a highly virulent strain of multi-drug resistant tuberculosis was found to be over-expressing the gene of *phoP* during a major disease outbreak (Soto, 2004). Nonetheless, the attenuation of virulence by deletion of the *phoP* gene has not affected the induction of wild-type *M. tuberculosis*-like immune response (Kamath *et al.*, 2005).

On the same base of attenuation, mutants for *M. tuberculosis*, *M. tuberculosis* mc^2 6020 vaccine and *M. tuberculosis* mc^2 6030 were produced. In mc^2 6020 vaccine, the genes encoding LysA which produce non-replicating *M. tuberculosis* and panC, D genes involved in lipid metabolism were deleted from *M. tuberculosis* genome, while in *M. tuberculosis* mc^2 6030 vaccine, the RD-1 gene region was deleted, and this means inhibition for ESAT-6, CFP-10 and other proteins encoded by this region. In spite of the fact that both mutants have been shown to be severely attenuated, their protective efficacy was found to be similar to BCG immunization (Kamath *et al.*, 2005). However, their evaluation in humans still involves problems with safety and stability.

Less virulent mycobacteria such as *M. microti*, *M. vaccae* or *M. smegmatis* that overproduce immunogenic antigens of *M. tuberculosis* are also another choice for producing vaccine against tuberculosis (Girard *et al.*, 2005). The vaccination of BALB/c and C57BL/6 mice by *M. microti* orally or by aerosol and subsequently challenging them via respiratory route with virulent *M. tuberculosis* gave a slightly better protection than BCG at oral dose of 100 million CFU (Helke *et al.*, 2006). However, the authors' claim must be viewed with some scepticism due to the massive dose required and the absence of a BCG vaccinated group which received the same high dose (Gupta *et al.*, 2007). Heat-killed *M. vaccae* is another vaccine from environmental mycobacteria which has been reported to stimulate Th1 response.

However, a high dose can only stimulate Th2 response, which is required for fighting against tuberculosis (Stanford, 1991).

Using environmental mycobacterium is not expected to be more effective than BCG since the first factor in BCG failure is losing specific genes for *M. tuberculosis* and these strains may be genetically different and less resemble *to M. tuberculosis* antigens, while the induction of CD8+ T cells may not appear as vital as in virtual *M. tuberculosis* infection.

Although live vaccines can provide sufficient and effective level of protection, safety concerns still hinder broad public acceptance of attenuation. For *M. tuberculosis* mutants as vaccine candidate, additional safety criteria such as verification of stable attenuation are still required. At least two no reverting independent mutations are strongly recommended for vaccines derived from *M. tuberculosis* before they could be considered for human trials (David *et al*., 2011).

Both rBCG30 and BCG ÄureC::hly recombinant live attenuated vaccines have entered clinical trials in the last decade. The development of rBCG30 has been achieved by the University of California. Preclinical models have demonstrated a better protection of rBCG30 over its parental strain and a Phase I clinical trial has proven its safety in adults. Two variants of the candidate vaccine were generated to limit its replication *in vivo*, thereby striving for increased safety in immunocompromised patients: (1) deletion of the *mbtB* gene rendering the strain dependent on exogenous mycobactin for iron acquisition, and (2) deletion of *panCD* conferring auxotrophy for pantothenate, vitamin B5. Although elevated efficacy and safety of rBCG30 variants over parental BCG have been achieved, the development of this candidate is currently on hold (Kaufmann, 2012).

Meanwhile, the vaccine candidate VPM1002 (BCG ΔureC::hly) induced better protection against TB in a preclinical challenge model. Superior protection of this particular vaccine over parental BCG is related to increased stimulation of different T cell populations involved in protective immunity. This is probably due to cross-priming as a consequence of elevated apoptosis of antigen-presenting cells harboring VPM1002. This recombinant candidate completed safety evaluation in adults in two Phase I trials and it is currently undergoing Phase II assessment in South Africa to determine its safety and immunogenicity in the target population (Kaufmann, 2012).

Live-attenuated vaccines offer a very potent means to prevent several human diseases, and a number of them are in routine use such as vaccines for polio, measles, mumps, rubella, and varicella. In general, these vaccines are safe, efficacious, and induce both local and systemic immune responses. While stimulating both humoral and cell-mediated immune (CMI) responses, live-attenuated vaccines also activate the innate and adaptive branches of the immune system. Although live vaccines offer great promises against intracellular pathogens, one obstacle to the development of these vaccines is the difficulty in achieving a satisfactory level of attenuation without severely compromising immunogenicity. The strength of the immune response is a function of the amount of the antigen expressed (Bastos & Borsuk, 2009).

Subunit Vaccine

Total cell wall, protoplasm and killed whole cell preparations were tested as experimental vaccine candidates for tuberculosis, including polypeptides and carbohydrates and lipids. Although the subunit vaccine candidate can be extracted from culture filtrates of *M. tuberculosis,* the emphasis is given to the proteins of *M. tuberculosis* surface such as the Ag85 mycolyl transferase family, the RD1 gene region proteins, ESAT-6 and CFP-10. After all, the filtrate alone contains more than 100 proteins revealed by proteomic analysis and theoretically has multiple protective antigens (Orme, 2006; Nicholas *et al.,* 2011).

The cell-mediated immune response, but not the humeral immunity, is essential for controlling intracellular pathogens including *M. tuberculosis*. Subsequently, the molecules inducing dominant T cell response and no B cell (antibody) response in *in vitro* immunological assays are the references for vaccine designer. IFN-γ is considered to be the most potential factor in protective immunity, as the IFN-γ gene disruption has led to fatal growth of bacilli in infected mice (Casadevall, 2003).

The Ag85 complex (A–C) consists of 30–32 kDa proteins' family that acts as mycolyl transferase. Ag85 has been used in two ways; first, as main vaccine with delivery vehicles such as liposomes and microspheres and the result of these studies showed reasonable protection from a high dose intravenous challenge. Second, Ag85 has also been used in boosting young mice in middle age with Ag85A after BCG vaccination. The result showed restoring to the waning immunity for *M. tuberculosis* challenge in old mice (Brooks *et al.*, 2001; Orme, 2006).

ESAT-6 is another candidate of tuberculosis vaccine. The importance of this protein is contributed by its presence in *M. tuberculosis*, but not in BCG. Some studies have shown ESAT-6 immunogenicity and it was found to have produced IFNγ responses in mice when it was mixed with potent adjuvant as MPL/DDA. However, the protection values in those studies were relatively small (Brandt *et al.*, 2002).

Fusion proteins or polyproteins (recombinant engineering vaccine) were first applied by Olsen (Olsen *et al.*, 2001) when a fusion protein made up of Ag85A and ESAT-6, and used to protect mice from *M. tuberculosis* challenge. The most important finding in Oslen's fusion protein is that the immunity sustained for at least 30 weeks and the vaccine could be given orally (Orme, 2006).

Mtb72F is a fusion protein constructed by Skeiky and his colleagues. This molecule consists of a polyprotein made up of Mtb32[C-terminal]–Mtb39–Mtb32 [N-terminal] of molecular size 72 kDa. Mtb72F in its native form successes in boosting BCG and stimulating strong IFNγ and CD8 CTL responses (directed to an epitope in the Mtb32-C component). The guinea pigs showed protection for over 50 weeks in similarity to BCG controls. Moreover, in the surviving animals, there were very few obvious granulomatous lesions and most of the lung tissues were clear (Orme, 2006; Skeiky *et al.*, 2004).

In the absence of reliable methods to predict the protective molecules, individual mycobacterial components (especially large number of proteins) are primarily screened for their immunoreactivity in terms of recognition by T lymphocytes and subsequent induction of IFN-γ

in vitro cultures. *In vivo* 'protective immunity' method by low dose infection animal models, followed by challenge experiments, is the standard protocol to evaluate the immunobiological features for individual antigens (Sable *et al.*, 2007a).

There are some features for the ideal candidate as tuberculosis vaccine can be considered before vaccine designing. It is believed that low molecular polypeptides can induce dominant Th1 response *in vitro* and they are recognized by T lymphocytes of animal and different human populations (Sable *et al.*, 2005b). The potential antigenicity of low molecular polypeptides may attribute to their stability and resistance to proteolysis process or because their small size render them more susceptible to proteolytic degradation, processing, intracellular trafficking and presentation (Skjot *et al.*, 2000). On the other hand, molecular mass proteins have failed to produce significant protection as compared to BCG and more than 30% of subunit proteins stimulating strong IFN-γ response (such as ESAT-6 and CFP-10, TB 10.4, TB 10.3) are not able to protect immunized mice from bacilli challenge, unless injected with multiple strong adjutants (Brandt *et al*., 2002). Moreover, EAT-6 has been observed to develop an active disease within a period of 2–5 years (Doherty *et al.*, 2002; Sable *et al.*, 2007a).

The hydrophobic or hydrophilic nature of a ligand and its homology with a receptor on the cell surface are important conditions for immunobiological activity through the interaction with class-I and class-II MHC molecules and induction T-cell responses (Doytchinova & Flower, 2001).

Structural stability with respect to the ability to induce an immune response is a potential factor for subunit vaccine. Many mycobacterial secretory proteins were found to be prone to degradation making them unacceptable as subunit vaccines. The failure of HSP-70 as a vaccine candidate is due to its instability and role in autoimmune diseases and immunopathology. On the other hand, some secretory proteins like HSP-16 and 19 kDa lipoprotein are highly stable due to the post-translational modifications (Sable *et al.*, 2007a). However, a possible negative aspect of stability is immunopathology as immunization with some of these long-lived molecules has been found to be correlated with immunopathology when it was used as experimental vaccines (Edwards *et al.*, 2001).

The post-translational modifications of mycobacterial proteins can positively or negatively affect in vaccine candidature by altering their immunogenicity or pathogenicity and this theory appears clearly in 45/47 kDa alanine-proline-rich antigen because its immunogenicity decreases with the changes in the glycosylation pattern (Romain *et al.*, 1999).

DNA Vaccines

A DNA vaccine stimulates immunity against an infectious disease by presenting particular genes or epitopes from an organism to the immune system (Nor & Musa, 2004). Wolff has found that expression of plasmid DNA carrying foreign genes is possible in mouse skeletal muscle (Istvan & Wolff, 1994). The produced protein is exposed on class I MHC molecules by endogenous pathway, followed by presentation to CD8 T cells (Kamar & Sercarz, 1996). Transfection of APCs is the second mechanism in DNA vaccine; however, limited number of DCs involves in transfection and priming immune response (Fine, 2001). Cross priming is the third mechanism in DNA vaccine. APCs receive antigen from myocytes or apoptotic cells and is subsequently presented to CD8 T cells (Sanjay *et al.*, 2000).

The use of a DNA vaccine offers many advantages in terms of stability, safety, and easiness in production and storage. The most important point, as shown in animal studies, is that DNA vaccines are able to induce both humoral and cellular immune responses (Montgomery *et al.*, 1997).

Furthermore, one DNA plasmid can yield many copies of the antigen over a prolonged period since antigens are endogenously expressed and presented on the MHC class I and activation of cytotoxic T lymphocytes (CTL), which is required against an intracellular pathogen like *M. tuberculosis* (Nor & Musa, 2004). However, the rate of protection for DNA vaccines is lower than that conferred by the BCG vaccine, so a cocktail of *M. tuberculosis* antigens to sustain protective immunity has been presented by some researchers as a solution for this particular drawback (Delogu *et al.*, 2000).

DNA vaccine can be constructed together with immunostimulatory molecules such as cytokine genes and immunostimulatory DNA sequences like CpG motif (unmethylated DNA sequence for build -in adjuvant) to enhance NK cell cytolytic activity and stimulate APCs since these molecules can polarize the immune response to Th1 (Kaerch *et al.*, 2002) and provide an indubitable protective immunity against TB. It has been suggested to immunize mice with IL-12 DNA plus the antigen of interest, as this combination can stimulate the immune system towards TH 1 cellular response and controlling the infection for a long term (Gurunathn *et al.*, 1998).

The other proposed improvement for DNA vaccine is incorporating granulocyte-macrophage colony-stimulating factor (GM-CSF). Although the addition of (GM-CSF) did not develop protective efficacy, increases in lymphoproliferation and INF production were detected (Kamath *et al.*, 1999).

Many suggestions have been presented to improve the plasmid DNA uptakes by the cells such as epidermal gene gun with DNA–coated gold particles. Unfortunately, this method induced type 2 immunity more than type 1, which is required against *M. tuberculosis.* Electrical pulse is another attempt to increase cell uptake for plasmid DNA (Mir, 2001). However, it is still unknown whether this method can actually increase immune protection.

According to the source of antigen, most of the genes used to produce vaccine against tuberculosis belong to *M. tuberculosis* such as Ag 85, 38kDa, Mtb39A, Pst-3, ESAT-6, Mtb 8.4, 72f and MPT-64,63,83, series and Ag85B,MPT-64+Ag85B, MPT-64+Ag85B+GMC-SF cocktails have shown some degree of protection for *M. tuberculosis.* However, other genes such as Hsp70, Hsp65 and 6kDa belonging to *M. leprae* also provide immunogenic protection to different models of infection (Taylor, 2003; Okada *et al.,* 2011).

Heat shocked protein (Hsp60) and Ag85A as DNA vaccines are important and this may attribute to the capability of these genes to elicit protection equals to BCG via induction TH1-type CD4 T cells and cytokines (Lowrie *et al.*, 1997; Taylor, 2003).

However, there are many factors to be evaluated in DNA vaccine before applying it in human level, especially in term of its safety, detecting the best method of injection, number of doses and finding the most antigenic and immunogenic epitops or by using cocktail of several mycobcterial antigens.

FAILURE OF BCG

BCG was produced in 1921. Ever since its early uses, the efficacy of the BCG vaccine has been questioned and an extensive number of trials have been carried out to analyze its performance (Agger & Andersen, 2002). Hence, understanding the reasons for failure in BCG may lead to identifying steps necessary for designing more effective vaccine.

Using BCG in all parts of world has led to high attenuating and as a result, differences in both phenotypic and genotypic levels, not only comparison with the original parents strain of BCG but also between the various daughter strains of BCG. The first theory, which may explain such difference, is the effect of mutations by losing a number of genes with potential relevance to protective immunity and this theory is supported by the variable protection level found in clinical trials using different strains of BCG (Behr *et al.,* 2002). The second proposed reason for the failure of BCG is the absence of specific T-cell antigen or BCG stimulates non-optimal blend of T cells; in another words, the lack of a particular induction of CD8 cells and subsequently insufficient level of immunity (Kaufman, 2001).

The final explanation for an inadequate protective effect of BCG is the human population's exposure in the tropical regions to large quantities of environmental mycobacteria, which may interfere with the efficacy of the BCG vaccination. Almost all defects of BCG such as the interfering of environmental mycobacteria and the absence of suitable antigen to T cells subset may be solved by using DNA vaccine (Agger & Andersen, 2002).

TUBERCULOSIS VACCINE STRATEGIES

The optimal tuberculosis vaccine may be achieved by understanding the limitations of BCG and designing a plane to overcome them.

BCG Lacks Important Antigens—exploiting genomics for rational vaccine development

Comparative genomic has revealed the differences between BCG and virulent *M. tuberculosis* as a number of regions designated RD1–RD16 and encompassing 129 open reading frames have been identified as absent regions in BCG vaccine. These genes are present in virulent mycobacteria but deleted during the attenuation and repeated passages of BCG (Brosch *et al.*, 2000). Some of these genes are likely associated with a virulence character and could play an important role in the failure of BCG; therefore, reintroducing selected genes from RD1–RD16 to BCG has been suggested as a way to enhancing the protective efficacy of the existing BCG vaccine. Hence, this study is an attempt to explore the capability of the RD regions as a possible candidate for TB vaccine.

RD1 (region of difference) is the best characterized region. Reintroduction of the RD1 into BCG has been done by using ESAT-6 in both protein and DNA forms and by mixing the Tcell inducer antigen with Ag85B to form subunit vaccine (Olsen *et al.*, 2001).

Another example of introducing missing genes was done recently using Ag85B and it was found to promote a level of protection greater than conventional BCG (Horwitz *et al.*, 2000). DNA immunization for Ag85B had also been applied successfully with MPT64, although this gene was found to be absent from some BCG vaccines (Morris *et al.*, 2000).

The opposite approach, i.e. knocking out genes from *M. tuberculosis*, has also been suggested as a way to create attenuated live mycobacterial vaccines with all the relevant protective *M. tuberculosis* antigens. McKinney *et al.* designed an isocitrate lyase knockout strain which multiplies like wild-type strains during the initial phase of its infection but this is rapidly eliminated in the chronic phase of infection (McKinney *et al.*, 2000).

However, safety concerns with genetically modified live vaccines are still very significant problems before these vaccines could become a reality. So far, many of these modified strains have turned out be too virulent. Moreover, none of the evaluated modified live vaccines has shown superior efficacy to the BCG vaccines (McKinney *et al.*, 2000).

Promoting the Optimal Blend of the T-cell Subsets — Targeting the CD8 T-cell Subset

An efficient level of CD8 cells is required for tuberculosis infection control. Disability of BCG antigens to gain access to the cytoplasmic compartment of host cells leads to poor CD8 cells stimulation. Many researchers have worked on inventing efficient antigen delivery systems to facilitate MHC class I-restricted immune responses. The most successful vaccine which stimulates acceptable level of CD8 cells has been done by Hess *et al.* via constructing a recombinant BCG strain secreting a pore-forming sulfhydryl-activated cytolysin (listeriolysin) from *Listeria monocytogenes* which provides an escape route from the phagosome into the cytosol of infected host cells (Hess *et al.*, 1998). Although listeriolysin-secreting recombinant BCG did not release from phagosomal vacuoles, an increased MHC class I presentation of cophagocytosed ovalbumin was observed (Agger & Andersen, 2002).

DNA vaccines have advantage of stimulating strong CD8 T-cell responses with both secretion of IFN-γ and cytotoxic activities. Meanwhile, 38-kDa lipoglycoprotein of *M. tuberculosis* and Mtb 8.4 DNA vaccine were found to induce a prominent CD8 T-cell response whereas antibodies against these antigens were absent (Fonseca *et al.*, 2001).

Prime-boost vaccination is another strategy to improving vaccine immunogenicity by inducing MHC class I-restricted responses. These studies are based on initial priming by DNA vaccine, followed by a boost with the antigen expressed in a viral vector system such as vaccinia virus. The primary results of these prime-boost vaccination schemes include enhanced immunogenicity and protective levels equivalent to BCG vaccine (McShane *et al.*, 2001).

Environmental Mycobacteria Interaction with BCG—design of Vaccines to Overcome the Influence of Sensitisation by Environmental Mycobacteria

There are several factors that cause low efficacy of BCG vaccine; these include temperature, ultraviolet radiation, and prevalence of environmental mycobacteria in tropical regions. The most important factor which can affect the BCG activity is the presence of environmental mycobacteria. There are some explanations for these drawbacks that are caused by this environmental mycobacteria. Among other, the exposure to environmental mycobacteria may mask the protection of a subsequent BCG vaccination. In other words, BCG can be totally eliminated after vaccination. Some animal studies have suggested that infection with environmental mycobacteria changes the immune reaction towards a detrimental humoral response, which could not be overridden by a subsequent BCG vaccination (Agger & Andersen, 2002).

In a recent study, Brandt and colleagues have inoculated mice with mycobacteria isolated from soil the samples in Karonga, Malawi, whereby BCG vaccination has been demonstrated not to protect against TB in a number of studies. Only weak immune responses after BCG vaccination were induced and these could be attributed by the inhibition of the initial BCG multiplication (Brandt *et al.*, 2002).

This finding clearly demonstrates that subunit vaccines are not influenced by the sensitization and stimulation of protective T cell response, while these factors could affect initial multiplication of BCG. This result may support the use subunit vaccine or DNA vaccine, which can provide enough level of protection against tuberculosis.

Preventing the waning of BCG efficacy—the potential of booster vaccination

BCG vaccine promotes high levels of immunity against childhood TB. However, the prevalence of TB increases with time, which means waning of BCG protection. This finding is observed by monitoring the increased number of pulmonary cases of TB in adolescence, which is not prevented by a BCG revaccination strategy. As the majority of the human population is already BCG vaccinated, an alternative strategy would be by boosting this existing immune response. Ag85 antigen has been reported as a successful booster to avoid the waning of immune response after BCG vaccination in mice. As opposed to live vaccines such as BCG and based on few selected antigens, a subunit vaccine can probably induce strong immune responses, which are not influenced by previous mycobacterial exposure (Brooks *et al.*, 2001).

GENOMIC DIFFERENCES BETWEEN *M. TUBERCULOSIS* AND *M. BOVIS* BCG

Continuous *in vitro* passaging of BCG leads deletion of specific regions from the genome of BCG and causes over-attenuation of the vaccine (Mostowy *et al.*, 2004). Comparative genome analyses of the *M. tuberculosis* genome and *M. bovis* BCG have shown that 16 genomic regions of *M. tuberculosis* are deleted or lacking in some or all strains of *M. bovis* and/or *M. bovis* BCG (Brosch *et al.*, 2000). Meanwhile, the loss of genomic regions during *in vitro* passaging is believed to have deleted not only the virulence factors but also certain key protective antigens in BCG (Behr, 2002).

Immunological evaluation of the proteins encoded by these regions was predicted to identify the antigens of *M. tuberculosis* which are important for developing specific diagnostic reagents and vaccines to control TB (Brosch *et al.*, 2000; Mustafa, 2005). These DNA sequences, designated as the Regions of Difference (RD1–16), are known to encode many putative molecules relevant for designing improved diagnostic and prophylactic strategies (Andersen *et al.*, 2000).

A few well-characterized proteins like ESAT-6, CFP-10, CFP-21 and MPT-64 are encoded by RD1 and RD2 of *M. tuberculosis* genome. Meanwhile, the loss of RD1 has been implicated to be the primary deletion that led to the attenuation of *M. bovis* and generation of vaccine strain BCG. Therefore, using the missing immunodominant RD antigens seems to be a promising strategy to produce potential vaccine against this deadly disease, "tuberculosis".

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