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Chapter 10: New Vaccines against Tuberculosis

Carlos Martín, Fabiana Bigi and Brigitte Gicquel

The current vaccine against tuberculosis (TB), bacille Calmette-Guérin (BCG), is a live vaccine derived from an attenuated strain of *Mycobacterium bovis*. BCG protects against severe childhood forms of the disease, but fails to protect against adult pulmonary TB in countries in which it is endemic. For more than 80 years, no new TB vaccine has successfully been developed. With TB eradication on the horizon, new vaccines with better protection than BCG are urgently needed.

The development of an effective TB vaccine seemed impossible until only a few years ago. In the last ten years of work with experimental laboratory models, many vaccine candidates have been developed. They include protein or DNA-subunit vaccines, modified BCG, and attenuated *Mycobacterium tuberculosis*. Some of these candidates are now being tested for safety and immunogenicity in human volunteers. For the first time, Phase I clinical trials of new TB vaccine candidates have started. Many of these new trials involve recombinant BCG or improved 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: they include those vaccinated with BCG, and those infected with *M. tuberculosis* or with HIV.

The goal is a new generation of vaccines effective against the transmissible respiratory forms of TB. Good candidate vaccines able to boost BCG, thereby improving protection, could be a reality in the short term. The second step is to obtain a new generation of vaccines able to replace the currently used BCG and make the eradication of TB feasible. These new vaccines can be expected in the middle term, and live vaccines are reliable and promising candidates. Indeed, these ultimate goals may require safe live vaccines.

10.1. Introduction

M. tuberculosis, the causative agent of TB, is one of the most successful human pathogens. In some areas of the world, TB has reached alarming proportions with a growing number of cases and deaths associated to human immunodeficiency virus/

acquired immunodeficiency syndrome (HIV/AIDS) (World Health Organization 2005). The emergence of cases of multidrug resistant TB (MDR-TB), which sometimes cause outbreaks, is a serious public health problem for any attempt to control the disease (World Health Organization/ International Union against Tuberculosis and Lung Disease 2004). *M. tuberculosis* is responsible for more deaths than any other single infectious organism; there are more than 8 million new cases and 1.7 million deaths annually. Control strategies for TB rely heavily on case detection and treatment with at least three different drugs over long periods of time. Consequently, the development of multidrug resistance is a serious impediment to any attempt to control this disease (Espinal 2001). No new drug has been added to the first-line treatment regimen for TB for more than 30 years. 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 (Toossi 2001). Indeed, TB remains the largest attributable cause of death in HIV-infected individuals, being responsible for 32 % of the deaths of HIV-infected individuals in Africa. In countries where the incidence of TB is highest, the populations most in need do not have access to treatment and, furthermore, in many cases anti-tuberculosis drugs are ineffective: the development of an effective TB vaccine is obviously now an urgent priority. Given the variable protective efficacy generated by the BCG vaccine against TB, there is a concerted effort worldwide to develop better vaccines that could be used to reduce the burden of TB.

10.2. Historical view

BCG is the only vaccine available for prevention of TB in humans. BCG is an attenuated live vaccine that was obtained after 230 successive passages in the laboratory between 1908 and 1921 from a pathogenic strain of *M. bovis*. It is an inexpensive vaccine that has been applied since the early '20s and it has been given to more than 2.5 billion people since 1948. It has a long-established safety profile and an outstanding adjuvant activity, eliciting both humoral and cell-mediated immune responses. It can be given at birth or at any time thereafter, and a single dose can produce long-lasting immunity. Recent studies with long-term follow-up of American Indians demonstrated that a single dose in childhood maintains immunization for up to 50–60 years after vaccination (Aronson 2004). However, different studies in other parts of the world have shown that protection provided by BCG wanes over time, and the efficacy of BCG in adolescent and adult populations is reported to be highly variable among different geographical regions (Andersen 2005).

BCG protects against severe forms of childhood TB, including miliary TB and extrapulmonary localization, such as the often fatal tuberculous meningitis. This is why BCG continues to be recommended in the vaccination calendar of the WHO (http://www.who.int/vaccine_research/diseases/tb/en) in countries with a high TB prevalence and incidence. BCG vaccination is currently compulsory in at least 64 countries and administered in more than 167. Indeed, BCG remains the most widely used vaccine in the world (Fact Sheets – BCG Vaccine, <http://www.cdc.gov/nchstp/tb/pubs/tbfactsheets/250120.htm>). In addition, BCG confers protection against leprosy and it has also been licensed as a treatment for bladder cancer.

The level of protection conferred by BCG is very variable: it differs according to the form of pulmonary TB and can be affected in those cases in which TB is associated with AIDS. The efficacy of BCG vaccines against pulmonary TB varies between populations, showing no protection in India but 50-80 % protection in the United Kingdom. The lack of protection against pulmonary TB in endemic regions has enormous importance from the point of view of public health as regards eradication of TB (Fine 1995). The reasons for the failure of BCG have been widely debated, and remain the 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 theory has been supported by the fact that exposure to environmental mycobacteria is prevalent in those countries where BCG confers low protection, and by a number of studies showing that exposure to environmental mycobacteria has an impact on the protection afforded by BCG in animal models (Buddle 2002, de Lisle 2005, Lozes 1997, Brandt 2002). This phenomenon has been proposed as a plausible explanation for the North-South gradient in the effectiveness of BCG (Brandt 2002). Host-related differences, such as genetic and host immune status, use of different BCG preparations, diverse levels of nutrition, and socio-economic issues should also impact BCG efficacy in different populations. It has been recently demonstrated that cross reaction is due to antigens shared between BCG and environmental mycobacteria (Demangel 2005). New vaccines deprived of major antigens shared with environmental mycobacteria will overcome the problem of the antagonistic effect of BCG to previous environmental mycobacterial exposure.

In parallel, neonatal vaccination with BCG has been reported as effective in reducing the incidence of childhood TB in endemic areas. The risk of disseminated BCG among adult AIDS patients with childhood BCG immunization is very low, and in addition, childhood BCG immunization is associated with protection of

adults with advanced AIDS against bacteremia with *M. tuberculosis*. Studies in Zambia have shown that bacteremia due to BCG or *M. tuberculosis* is rare among children who have BCG immunization (even recent) and symptomatic HIV infection (Waddell 2001).

10.3. Genetic diversity between BCG vaccines

Since 1921, when BCG was used for the first time, different laboratories throughout the world have continued to sub-culture BCG, giving rise to the appearance of different variants, such as BCG Pasteur, BCG Moscow or BCG Brazil. 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 because differences in protection and effectiveness could be due to variations between strains, and for this reason, the WHO has recommended lyophilization of BCG vaccine stocks and storage at -80°C (Behr 2002).

BCG vaccines have been classified into two major groups. BCG Tokyo, Moreau, Russia, and Sweden secrete large amounts of the MPB70 gene, have two copies of the insertion sequence IS6110, 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 IS6110, and do not contain the methoxymycolate and MPB64 genes (Ohara 2001).

Comparative genomic analysis has revealed the existence of several *M. tuberculosis*-specific regions that have been deleted from BCG with the loss of more than 100 genes (Behr 1997). These genomic comparisons have made it possible to determine the order of genetic events, including deletions and duplications, and changes in the IS6110 copy number which occurred between its first use in 1921 and 1961 (Behr 1999). 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 sub-strain 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.

10.4. New vaccines: from the bench to clinical trials

Even if BCG has been demonstrated to be extremely useful and at the moment is the most utilized vaccine in the world (World Health Organization 1995), the development of new vaccines against pulmonary TB, which are able to replace the current BCG vaccine, is an important challenge (Kaufmann 2005). Since humans are the only reservoir of *M. tuberculosis*, the development of vaccines more effective than BCG could make TB eradication possible [see Development of New Vaccines for Tuberculosis Recommendations of the Advisory Council for the Elimination of Tuberculosis (ACET) on the Internet <http://www.cdc.gov/mmwr/preview/mmwrhtml/00054407.htm>].

The lung is the portal of entry of *M. tuberculosis* in most human infections and provides a suitable environment for this slowly replicating pathogen. Infection is established in alveolar macrophages of the distal alveoli before it is recognized 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, which migrate from the site of the infection in the alveoli to the draining lymph nodes. The development of interferon-gamma (INF- γ)-secreting CD4+ T cells is dependent on the secretion of IL-12 by infected dendritic cells. Subjects deficient in receptors for INF- γ and IL-12 are extremely susceptible to mycobacterial infections, confirming the absolute requirement for T helper 1 (Th1)-like T cells for host immunity (Flynn, 2004).

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 adjuvants or live vaccines capable of inducing potent T-cell responses have been developed and some have entered clinical testing.

10.4.1 Challenges for tuberculosis vaccine development

There are a number of substantial underlying problems to be faced in developing vaccines with enhanced protective efficacy against TB (Table 10-1). In contrast to a classical vaccine-preventable disease such as smallpox, recovery from infection

with *M. tuberculosis* is not associated with sterilizing 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 (Caminero 2001). Therefore, vaccines need to be more effective than infection with *M. tuberculosis* itself (Van Rie 1999).

Table 10-1: Major challenges and concerns for TB vaccine development (modified from Martin 2005)

CHALLENGE	CONCERN
One third of human population infected with TB	New vaccines should be preventive and immunotherapeutic, too
Co-infection HIV/ TB	Safety: vaccines should be as attenuated as, or even more attenuated than BCG
Large percentage of population vaccinated with BCG	New vaccine candidates should be tested in a BCG-vaccinated population

A third of the population worldwide is estimated to be infected with *M. tuberculosis*. Therefore, any new TB vaccine should protect pre-exposure people from developing infection, as well as post-exposure, latently infected, healthy individuals from developing the disease, or should be used as an immunotherapeutic 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 immunized 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 (Vuola 2003).

Advances in the characterization of genes and antigens of *M. tuberculosis* and the technological development (Clark-Curtiss 2003), with the help of the genome sequences of different mycobacterial species (Cole 1998), have provided insights into the tubercle bacillus (see TubercuList Web Server on the internet <http://genolist.pasteur.fr/TubercuList/>). In addition, the current progress of mycobacterial genetics has made the inactivation of selected genes possible, allowing the rational attenuation of *M. tuberculosis* (Pelicic 1997). Finally, the improvement in comprehension of the basic immune mechanisms involved in TB has considerably contributed to the rational design of the next generation of vaccines. Remarkably, novel immunological concepts about the mechanisms underlying memory and regulation of the immune response against TB have been defined as relevant for the

rational design of new-generation vaccine candidates (Kaufmann 2005). Therefore, this progress in the different fields of TB research has placed us in a better position for the construction of new effective and safe vaccines against TB.

Many 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 (see EC TB VAC consortium on the internet <http://ec.europa.eu/research/press/2004/pdf/pr2304-tb-vac.pdf>) (Orme 2005, Martin 2005, Kaufmann 2000). As a result of this basic research, the enormous effort of the scientific community in the last 10 years has generated a great number of vaccine candidates against TB to be tested in different laboratory experiments, experimental animal models (Williams 2005, Orme 2006), and clinical trials in human populations (Skeiky 2006).

Broadly, two approaches have been used to improve the TB vaccine. The first involves subunit vaccines that can deliver immunodominant mycobacterial antigens. Both protein and DNA vaccines induce partial protection against experimental TB infection in mice but their efficacy has generally not been better than that of BCG (Huygen 1996). New antigen formulations, including multiple antigens or epitopes, are under investigation and it is hoped that they will afford better protection in humans. The second approach involves 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 (Britton 2003).

10.4.2. Animal model for vaccine preclinical trials

The most commonly used animal model is the mouse, followed by the guinea pig. Primate models have also been developed and are being used as an important testing model prior to clinical trials (Langermans 2001).

The advantage of the mouse model comes from the amount of reagents and genetic information available, and its logistical and economical advantages, in comparison with other models such as the guinea pig. Mice have a certain tolerance to this infection; it triggers a moderate inflammatory reaction that allows the control of the bacillary concentration at a low level, without eradicating it. The commonest route of infection is intravenous, because this switches on acquired immunity very rapidly. The experimental model induced by aerogenesis, uses the most physiologically infectious route and at the same time is more aggressive for the host than intravenous administration. This happens because the induction of immunity is

quicker after intravenous inoculation than after aerosol. Both models have demonstrated that immunity against infection is based essentially on the stimulus of a Th1-type response, that is to say, in the stimulation of CD4⁺ T cells able to produce IFN- γ and to activate the infected macrophages (Orme 2001, Aguilar 2006). Testing the protection obtained from new vaccines using the guinea pig model has become a compulsory experiment because of the extreme sensitivity that this animal has demonstrated with *M. tuberculosis* inoculation, and the toxic response generated. This has allowed the comparison of different TB vaccine candidates (Williams 2005). On the other hand, the necessity to evaluate the protection of any new vaccine in an experimental model that is physiologically closer to humans, before carrying out human clinical trials, has led to the development of the primate model (Langermans 2001, Langermans 2005).

10.5. Subunit vaccine candidates

Due to safety reasons, non-viable sub-unit vaccines are the first to be considered for human trials. Subunit vaccines have been selected by various rational and experimental approaches (Table 10-2). Results with non-viable subunit vaccines are encouraging but their protective effects have to be at least equivalent to that of BCG before they can be considered for testing in humans.

Potential TB subunit vaccines have been obtained by using immunodominant TB antigens, for example ESAT-6 [6-kiloDalton (kDa) early secretory antigenic target], which confers some degree of protection against *M. tuberculosis* in mice (Olsen 2004) and recently in non-human primates (Langermans 2005).

A fusion protein based on ESAT-6 and antigen 85B administered to mice together with a potent adjuvant induced a strong dose-dependent immune response. This immune response was accompanied by protective immunity comparable to BCG-induced protection over a broad dose range. The vaccine induced efficient immunological memory, which remained stable at 30 weeks post vaccination. More recently, it has been documented that the synthetic adjuvant IC31 augmented the immune response and protective efficacy of the combination of Ag85B-ESAT-6 in the mouse aerosol challenge model of TB (Agger 2006).

Table 10-2: TB vaccine candidates tested in humans (modified from Martin 2006)

Vaccine type	Definition	Stage of development	Pharmaceutical company or research group
Sub-unit			
72f	Selected antigens identified from human response	Phase I trial ready for phase II BCG boosting strategy	GlaxoSmithKline (EU/TBVac/Aeras) (Irwin 2005)
85B-ESAT6	Recombinant major antigens	Phase I trial BCG boosting strategy	SSI (EU/TBVac) (Langermans 2005)
Viral vector			
MVA-85A	Recombinant modified vaccinia virus Ankara Ag85A	Phase I trial BCG boosting strategy	Oxford University, United Kingdom (EU/TBVac) (McShane 2004)
Live vaccines			
rBCG30	Recombinant BCG: over expression of Ag85B	Phase I trial	(UCLA/NIH /Aeras) (Horwitz 2003)

EU/TBVac: <http://www.tb-vac.org>

SSI: <http://www.ssi.dk/sw1404.asp>

UCLA: www.research.ucla.edu/tech

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)). Immunization 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 immunization resulted in the protection of C57BL/6 mice against aerosol challenge with a virulent strain of *M. tuberculosis*. Most importantly, immunization of guinea pigs with Mtb72F produced a prolonged survival (> 1 year) after aerosol challenge with virulent *M. tuberculosis*, comparable to BCG immunization. Mtb72F in the AS02A formulation is currently in phase I clinical trials, making it the first recombinant TB vaccine to be tested in humans (Skeiky 2004, Irwin 2005).

10.6. Subunit vaccines for boosting BCG

Since acellular vaccines have never been demonstrated to confer better protection than BCG in preclinical testing, they have been proposed to be used for boosting BCG. Heterologous prime-boost immunization strategies can evoke powerful T cell immune responses and may be of value in developing an improved TB vaccine. Importantly, this regimen of vaccination expands pre-existing memory T cells against antigenic epitopes shared by BCG and the booster vaccine. Experiments using protein subunits in animals previously vaccinated with BCG have given very good results (Brooks 2001). These experiments used Ag85A, because it was previously demonstrated that most CD4⁺ T cells accumulating in the lungs of memory-immune mice after challenge recognize this antigen. This vaccine strategy may have applications in the prevention of reactivation of TB in the elderly.

Enhanced immunogenicity and protective efficacy against *M. tuberculosis* has been demonstrated for BCG after boosting with a recombinant modified vaccinia virus called Ankara. The recombinant 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 lymph nodes in the lung (Goonetilleke 2003). This vaccine was tested for the first time in humans (McShane 2004).

Similarly, a combination of vaccines has been shown to be more protective in preventing bovine TB in cattle than single vaccines. Tested in calves, prime-BCG boost strategies of vaccination were reported to induce cellular immune response (Vordermeier 2006) and high levels of protection against challenge with virulent *M. bovis* (Cai 2006, Skinner 2003).

10.7. Recombinant BCG vaccines

Recombinant BCG (rBCG) techniques may be useful for the development of a more effective mycobacterial vaccine than the parent BCG now in use. Various strategies have been used to develop recombinant BCG against mycobacterial diseases (Table 10-3). 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 of the immunodominant TB antigens. rBCG vaccine (rBCG30), which expresses and secretes the 30 kDa major secreted protein of *M. tuberculosis*, also referred to as a-antigen and antigen Ag85B (Horwitz 2000), is associated with better host survival after challenge than parental BCG in the highly demanding

guinea pig model of pulmonary TB. Animals immunized with rBCG30 and then challenged with an aerosol of a highly virulent strain of *M. tuberculosis* survived significantly longer than animals immunized with conventional BCG (Horwitz 2003, Horwitz 2005).

Alternatively, BCG genes that have been lost by deletion from the parental *M. bovis* strain, and that are important antigens, can be restored. An example is the case of ESAT-6 deleted from region RD1 (region-of-difference 1) of BCG (see Unité Génétique Moléculaire Bactérienne on the internet <http://www.pasteur.fr/recherche/unites/Lgmb/Deletion.html> (Pym 2003). Both these approaches are attractive for improving or adding antigens to BCG and could be important for conferring immunity against TB (Table 10-3).

Table 10-3: Live tuberculosis vaccine candidates in advanced preclinical testing (modified from Martin 2006)

Vaccines	Definition	Research group
rBCG:RD1	Recombinant BCG RD-1 of <i>M. tuberculosis</i> introduced	Institut Pasteur Paris, France (EU/TBVac) (Pym 2003)
rBCG- Δ ure-hly	Recombinant BCG with BCG urease gene deleted and listeriolysin of <i>Listeria monocytogenes</i> introduced	Max-Planck Institute Berlin, Germany (EU/TBVac) (Grode 2005)
<i>M. tuberculosis</i> <i>phoP</i> mutant	Rational attenuation of clinically isolated <i>M. tuberculosis</i> by deletion of virulence gene	Zaragoza University, Spain Institut Pasteur (EU/TBVac) (Martin 2006)
<i>M. tuberculosis</i> auxotrophic mutant	Rational attenuated <i>M. tuberculosis</i> H37Rv by <i>lysA</i> and <i>panCD</i> deletion	Albert Einstein College of Medicine, New York, USA (NIH) (Sampson 2004)

A second strategy involves enhancement of the relatively low intrinsic ability of BCG to induce the CD8⁺ T cell response. This type of rBCG has the capacity to alter the permeability of the membranes of phagosomes in host cells and gain access to cytoplasm. Major histocompatibility 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 is a pore-forming sulfhydryl-activated cytolysin essential for the release of *Listeria monocytogenes* from phagosomal vacuoles into the cytoplasm of host cells, thereby facilitating presentation of antigens by MHC class I molecules. Hess and collaborators constructed an rBCG, which secreted biologically active

listeriolysin (hly^+ -rBCG), shown to improve MHC class I-presentation of co-phagocytosed soluble protein (Hess 1998). Tested in mice, hly^+ -rBCG elicited better protection against aerosol infection of *M. tuberculosis* than the parental BCG (Grode 2005). In addition, a version of a hly^+ -rBCG, deficient in urease C, has been shown to significantly improve the level of protection against *M. tuberculosis* in mice and to increase apoptosis in infected macrophages. Urease deficiency enables acidification of the phagosome so that listeriolysin finds its optimum pH for perforation of the phagosomal membrane. The authors advocate that the high efficacy observed may be due to the presentation of extracellular antigens with the MHC class I molecules to CD8⁺ T cells (cross-priming) caused by apoptosis (Grode 2005).

In another approach, rBCG has been constructed to secrete diverse cytokines, including IL-2, IFN- γ , and others, in an attempt to enhance the immuno-stimulatory properties of BCG (Murray 1996).

Additionally, 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 effective rBCG multivalent vaccines to control major infectious diseases. Promising rBCG vaccines against a variety of viral, bacterial and parasitic diseases have been shown to induce protective immune responses in murine and primate challenge models (Santangelo 2007, Ohara 2001, Winter 1995).

10.8. Live vaccines based on attenuated *M. tuberculosis*

Of the six immunodominant antigens of *M. bovis* (ESAT-6, CFP10, Ag85, MPB64, MPB70, MPB83), five are either deleted from or down regulated in some or all BCG strains. Moreover, RD1, a region of difference between *M. tuberculosis* and BCG (see the Annual report of Bacterial Molecular Genetics for 2002 on the internet <http://www.pasteur.fr/recherche/RAR/RAR2002/Lgmb-en.html>) includes two of the six immunodominant antigens ESAT-6 and CFP10, which have been shown to be important for protection against *M. tuberculosis* challenge in the guinea pig model (Pym 2003). The advantage of rational attenuated *M. tuberculosis* as a vaccine is that hundreds of genes deleted from BCG as a consequence of the progressive adaptation of BCG strains to laboratory conditions are still present in *M. tuberculosis*. The advances in TB research and the completion of the *M. tuberculosis* genome sequence (Cole 1998) have facilitated the analysis of the contribution of individual genes to the virulence of *M. tuberculosis* (Camacho 1999).

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 (Perez 2001) and exhibits impaired multiplication *in vitro* within mouse-cultured macrophages; it is also attenuated *in vivo* in a mouse infection model. Thus, the PhoP gene might be involved in the regulation of complex mycobacterial lipids implicated in the virulence of *M. tuberculosis* (Gonzalo Asensio 2006). Results in an animal model make a *phoP* mutant a promising TB vaccine candidate (Martin 2006). Similarly, it was recently demonstrated that the lack of *mce* (mammalian cell entry) gene expression in *M. tuberculosis* decreases virulence and increases immunogenicity, providing better protection than BCG against TB in the mouse model (Aguilar 2006) (Table 10-3).

Auxotrophic mutants, which require the addition of nutrients for survival, maintain their infective ability, but have a limited replication in the host. These vaccines are attenuated to different degrees and have diverse potential as vaccine candidates, as assessed in animal models (Martin 2006, Smith 2001).

There are major issues associated with the use of live organisms, especially safety and regulatory hurdles, that need to be overcome, in particular with attenuated *M. tuberculosis*. The early use of BCG was marked by a tragic accident. In 1927, in Lubeck, Germany, more than 25 % of approximately 250 infants who received a batch of the vaccine developed TB. It was later recognized that this batch was accidentally contaminated with a virulent strain of *M. tuberculosis* (Kaufmann 2006).

In a world conference held in Geneva in 2005, a consensus document was elaborated on, aiming to promote the movement of the most promising vaccine candidates to the clinic and towards control of TB (Kamath 2005). A set of criteria were proposed to be considered during the vaccine development process. One of the criteria for a live candidate vaccine is the presence of at least two non-reverting independent mutations on the mycobacterial genome. In this regard, double auxotrophic mutants have recently been described (Sampson 2004, Sambandamurthy 2005, Sambandamurthy 2006). Some of these live vaccine candidates elicited protective immune responses similar to that of BCG in mice, and better than BCG in guinea pigs (Martin 2006, Williams 2005). These findings are encouraging, and further studies in non-human primates should be performed.

Live vaccines have been questioned because of the failure of the BCG vaccine due to pre-exposure to environmental mycobacteria, which was shown to block multiplication of BCG and induction of protective immunity in animal studies. Evidence was provided that sensitization with environmental mycobacteria may have a direct antagonistic effect on BCG vaccination (Flaherty 2006). Recently, it was experi-

mentally demonstrated (Demangel 2005) that cross-reaction is due to antigens shared between BCG and environmental mycobacteria, such as Ag85B, but not deleted antigens of BCG, such as ESAT-6 and CFP10. These results strongly suggest that prior exposure to live environmental mycobacteria primes the host immune system against mycobacterial antigens shared with BCG, and that recall of this immune response on vaccination results in accelerated clearance of BCG and hence decreased protection against TB. The authors demonstrated that the persistence of BCG *in vivo* could be markedly augmented by the stable insertion of RD1, which encompass *esat 6* and *cfp10* genes.

Rational attenuated *M. tuberculosis*, which includes regions deleted in BCG with major antigens not shared with environmental mycobacteria, will probably overcome the problem of the antagonistic effect of BCG to previous environmental mycobacterial immunization. *M. tuberculosis* mutant vaccine candidates have to induce long-term cellular immune responses, essential for effective protection against TB. New live vaccines should be stored lyophilized, and current technology allows monitoring of any possible variations of genomic composition by comparative hybridization experiments using DNA microarrays.

10.9. Conclusions

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 because it is effective against the severe forms of TB and its use prevents 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 sub-strains may provide an answer to this important question. The World Health Organization (WHO) and the International Union Against Tuberculosis and Lung Disease (IUATLD) could then identify the BCG sub-strains that provide the best protection and recommend them for future vaccination worldwide (Corbel 2004).

For many years, the discovery of new TB vaccines effective against pulmonary TB has been considered an elusive quest, but the TB vaccine field has blossomed in the last decade. Research to develop improved TB vaccines seems to be at a decisive point in 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. 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” organized by the WHO and the IUATLD, a joint effort was established involving diverse governmental organizations in Europe (FP5 and FP6 Framework Programmes) and the USA by NIH, and recently by the AERAS Foundation.

For the first time, after 80 years of widespread use of BCG, evaluations of new candidates in humans are available including recombinant vaccine virus (Table 10-2). Nevertheless, the development of a new vaccine conferring better protection than BCG, and able to replace it, remains a challenge for the scientific community. If eradication of TB is to be possible and affordable, 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 BCG. However, in order to confer the complex immunity required to protect against TB, it is possible that more than single antigens will be necessary. Progress to date with live attenuated *M. tuberculosis* vaccines indicates that it is possible to design strains that are highly attenuated, even for immunodeficient animals. These “classical” vaccine candidates have to mimic natural infection as closely as possible without causing disease (Young 2003).

The goal of evolving an effective licensed vaccine by the year 2015 has been proposed by Stop TB/WHO. It is estimated that at least 20 vaccine candidates should enter Phase I safety trials, with around half going forward for immunological evaluation in Phase II trials, leading to four Phase III efficacy trials (Young 2006). Vaccination is expected to make a major contribution to the goal of eliminating TB worldwide by 2050. Still, developing a new effective vaccine will require innovation in scientific research, a proactive approach to clinical trials of new vaccine candidates and application of vaccines as a part of an integrated approach to disease control (Young 2006).

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