Children can be deprived of their right to education, ostracized by their peers and sometimes by teachers, due to having a family member sick with TB

Sense – Lisbet Fernández
Installation
Clay and glass
(Variable dimensions)

'The word was not listened or I didn't say it'

La marcha **(Poem) – Dulce Maria Loynaz**

Lisbet Fernández
Camagüey, Cuba

Lisbet Fernández paints big format portraits with children as her central subject.

The Myto Gallery exhibited her collection entitled 'Con los ojos abiertos', which brings together her latest works, consisting of five life-size pieces about children, made of clay and located in different contexts.

She participated in the exhibition 'Escultura Transeúnte' (Street Sculpture), organized by CODEMA in 2005 and in the exhibition 'Doble Blanco', collateral to the 9th Havana Biennale, together with artists Alain Pino, Iván and Yoán Capote.

CHAPTER 14

Subunit Vaccines against Infection with *Mycobacterium Tuberculosis*: Preclinical and Clinical Considerations

Jes Dietrich and T Mark Doherty

Introduction

M. tuberculosis, the causative agent of TB, remains a major worldwide health problem that causes more than two million deaths annually. The disease can be cured by antibiotic treatment but the difficulty of timely diagnosis, socio-economic factors in TB-endemic areas, and the fact that bacterial clearance requires many months of treatment have combined to prevent successful global TB control by antibiotics. Vaccination has also been only partially successful, despite the fact that the only current vaccine against MTB, *M. bovis* BCG, is the most widely used vaccine in the world. This is because it only provides protection against the disease for a limited number of years (1–3): while it has clear beneficial effects against TB in childhood (3–6), in highly TB-endemic regions, where exposure is continuous, infection in adults is all but universal. Therefore, a new vaccine is urgently needed that can prevent new cases of TB—estimated at 8–10 million a year (7). Further, an estimated two billion people are already infected with MTB (8) and many of these people will develop disease in the future if not treated, contributing to the complexity of TB control efforts. This needs to be considered in future TB vaccine design and development.

As with any vaccine development programme, there are preclinical and clinical considerations, both of which will be discussed below.

Preclinical Considerations

A subunit vaccine needs to consider the complexity of MTB's lifestyle. Exposure to MTB often results in lifelong infection due to a large range of evasion mechanisms deployed by the bacterium. The acute phase of MTB infection is characterized by rapid bacterial growth and the development of an initial immune response dominated by recognition of secreted bacterial antigens (9–11). Macrophages and lymphocytes migrate to the site of infection, resulting in formation of granulomas in the lungs. In the majority of cases, the infection is brought under control by the immune system. Cytokine production by Th1 lymphocytes is known to be an essential factor in this process (12–15). However, the bacterium is not always eliminated but adapts to the hostile environment of the host and enters a stage (often referred to as dormancy or latency) characterized by drastically altered metabolism and a significant change in gene expression (16–19). It is unclear at present if the bacteria in this stage is truly dormant, or persists through limited but continuous replication. It likely exists in a continuum of active and less active forms (20). The outcome is a latent stage of infection without clinical symptoms that may last for many years or even decades. Latency is a dynamic process in which bacterial outgrowth is controlled by the immune response, but this balance can change at any point in time (e.g. immunosuppression by HIV or during therapy), leading to rapid bacterial replication and clinical reactivation of TB (21–24). If the disease reactivates, the bacteria can be transmitted to other individuals. Considering the phenotypic change of the bacterium during the different stages of TB infection, it is most likely that a successful vaccine against TB may need to induce immune recognition of a broad spectrum of bacterial antigens.

Why Change the Current Vaccine, BCG?

Since 1921, when BCG was introduced, more than three billion people have received the vaccine. BCG is cheap and safe and protects children (the most susceptible group) efficiently against the early manifestations of TB (3–6). However, BCG has proven to have limited effect against adult pulmonary TB, particularly in the developing world, and estimates of protection have ranged from 0 to 80 per cent based on large, well-controlled field trials (25–27). Although still a subject of debate, most studies have reported that BCG is protective for, at most, 10–20 years (1–3). The timeframe for the waning of BCG-induced protection through childhood and young adult life coincides

with the gradual increase in TB incidence, which in some highly TB-endemic regions, such as sub-Saharan Africa, reaches a peak of >500 cases per 100,000 individuals in the 25–35-year-old age group.

Recent studies have indicated that BCG is ineffective in individuals pre-sensitized to mycobacteria, for example, by exposure to environmental mycobacteria or prior BCG vaccination or MTB infection (28). BCG is a live vaccine and the development of protective immunity after BCG vaccination appears to require vaccine replication in the host, which can be prevented by a pre-existing immune response that can cross-react to BCG or antibiotic treatment (29–31). The failure of BCG in sensitized individuals means that that BCG cannot be used as a booster vaccine to counteract the waning effect of the BCG vaccination given after birth—as attested to by the failure of attempts to boost protection by giving multiple doses of BCG (32, 33). On a global scale, widespread latent TB infection in adults is moreover a significant barrier to attempts to boost immunity. Therefore, a novel vaccine to replace (or improve) BCG faces not just one but many daunting technical problems.

Making a Subunit Vaccine against MTB: Challenges for Vaccine Developers

While the antigens used in subunit vaccines are crucial, it is important to stress that any vaccine against infection with MTB should induce the correct response against the antigens used. This is particularly important since it is thought that MTB has developed the ability to divert the immune response away from that which gives optimal protection and to change its protein expression according to the immune pressure that it is under—including the expression of proteins to directly interfere with the host's immune response and so-called decoy proteins.

A major component of MTB's success as a pathogen rests on its ability to survive within host cells—especially immune cells such as macrophage/monocytes, which are charged with both killing bacteria directly by phagocytosis and priming immune responses by antigen presentation. MTB does this by interfering with the process of macrophage activation and phagocytosis at virtually every stage. One obvious result is the disruption of phagosome maturation, creating an intracellular compartment that lacks the acidic, hydrolytic environment needed to kill the bacteria. However, fusion with other vesicles and membrane remodelling and trafficking still occurs, allowing MTB to acquire necessary nutrients and export its own proteins (34–36). A wide range of genes is involved in this process. The functions of some are as yet unknown, but putative transporters, iron scavenging molecules, and lipid synthesizing molecules are all apparently important (37) in preventing normal phagosome maturation. Some lipids, such as Trehalose dimycolate (TDM), can

also prevent phagosome maturation (38) while others, including ManLAM (39), seem to be mimics of host phosphatidylinositols, whose presence on the surface of the vacuole normally indicate maturation state (40, 41). Likewise, the expression by MTB of a eukaryotic-like serine/threonine protein kinase G can inhibit phagosome-lysosome fusion, suggesting that interfering with host signal transduction mechanisms is a major survival strategy for MTB (42).

This modulation of host responses goes beyond intracellular trafficking and has obvious implications for vaccine design. It has been suggested that invasion of phagocytes which are not yet activated is important for the bacteria's survival: exposure of macrophages to IFN-γ and/or TNF-α before—but not after—infection decreases the ability of pathogenic mycobacteria to inhibit phagosome maturation and function (36, 43). Activating these responses has thus been a major goal for the vaccines under development. However, merely establishing such responses may not be sufficient: MTB can react to the host immune response in multiple ways. The first of these is by directly countering the Th1 response. Live bacteria or MTB cell wall extracts can inhibit some of the downstream effects of IFN-γ by reducing STAT-1 binding to transcriptional activators (44)—consistent with reports that in vitro IFN-γ recall responses are reduced in patients with advanced TB (45–47) while IL-4 is elevated (48–51), and the observation that these responses move in opposite in most patients during therapy suggests that this state is directly related to the disease (52–55). The mechanism remains incompletely described but several potential virulence factors have been identified. Phosphoglycolipid (PGL), a cell wall component which induces IL-4 and IL-13 production, is a possible contributor and its expression has been associated with virulence in clinical strains (56–59). Another potential immunosuppressant is LAM—a major cell wall component of MTB—which can bind to the Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) molecule, expressed on the surface of dendritic cells (DC), inhibiting DC maturation and IL-12 production and inducing DC to secrete IL-10 (60, 61). Consistent with this, studies have found that expression of IL-10 is significantly elevated in TB patients with active disease (45, 62). Similarly, the 19 kDa lipopotein of MTB interacts with host antigen-presenting cells via TLR1/2 (63) leading to inhibition in cytokine production, antigen-processing, MHC II expression, and immunity (64–67). Since TLR2 is implicated in the inflammatory response to mycobacteria (68, 69), it has been suggested that inhibition of antigen presentation and response to IFN-γ by the 19 kDa molecule may allow the bacteria to evade immune surveillance during the latent phase of infection (65, 66, 70).

Other MTB genes, such as *nuoG*, appear to interfere with the ability of the host to remove infected cells by apoptosis, another important route of controlling intracellular pathogens. Knock-ins of this gene conferred on

avirulent mycobacteria both the ability to inhibit apoptosis and increased virulence in mice, while its deletion rendered MTB less able to inhibit apoptosis of infected human monocytes (71).

Finally, it has been suggested that MTB attempts to avoid the consequences of the immune response induced by expressing 'decoy' molecules, which stimulate immune responses which are antigen specific but ultimately ineffective. For example, immune responses to the 27 kDa lipoprotein of MTB are characterized by strong IFN-γ secretion but in animal models, at least, these responses are not protective and, in fact, appear to promote bacterial growth (72). The same is true of responses to the highly polymorphic Pro-Glu-polymorphic GC-rich sequence (PE-PGRS) and Pro-Pro-Glu-major polymorphic tandem repeat (PPE MPTR) gene families (73–75).

Taken in total, these studies indicate that MTB is able to interfere with almost every stage of the host's immune response and give some insight into why it is such an effective pathogen. As mentioned above, countering these complex strategies in the design of novel vaccines is a daunting task requiring the activation of the correct response against the correct antigenic targets.

Subunit Vaccine Strategies against MTB

Priming Versus Boosting Vaccines

The vaccines being developed fall into two camps. The first is vaccines aimed at replacing BCG and giving longer, or more effective protection. At the present time it is thought unlikely that a subunit vaccine can replace BCG, and this vaccine strategy is therefore mostly focused on recombinant BCG or attenuated MTB vaccines. The second strategy involves vaccines designed to be given to already BCG-vaccinated individuals to further boost (and hopefully prolong) the BCG-induced immunity. Compared to recombinant mycobacterial vaccines, where it is unclear whether such an attenuated vaccine is virulent enough to overcome the existing immunity due to earlier exposure to environmental mycobacteria or a prior BCG vaccination, subunit vaccines do not have this concern. Therefore, the obvious choice is to use the mycobacterial vaccines for priming, and subunit vaccines as boosters, allowing designers of boosting vaccines to take advantage of the prevalence of BCG vaccination and the likelihood that this will persist, at least for the foreseeable future. However, since a vaccine administered as a booster to adolescents or older children may also be given to individuals who did not receive the BCG vaccine or who received an ineffective BCG vaccination (incorrectly administered, or with vaccine that was too old or incorrectly stored), a booster vaccine should also be able to prime an effective immune response. As a result, all of the vaccines currently in clinical trials were initially screened in animal models

for the ability to prime a protective immune response at least as efficacious as BCG (76). Several vaccine strategies involving BCG and subunit vaccines are presently being pursued (as discussed below).

Since booster vaccines by definition will be given later in life, the assumption that two billion people are latently infected with MTB means any booster vaccine will also of necessity be given to large numbers of latently infected individuals unless prior screening is given. This raises the question of safety and any such vaccine will need to be rigorously screened for safety in MTB-infected individuals. However, it also raises the possibility—can we do anything for people who are already infected, either because they did not receive a primary vaccination, or because it failed? Mathematical modelling suggests that a post-exposure vaccine effective at preventing disease in latently infected individuals would cause a significant decrease in the number of new cases in the short term, but that over time a pre-exposure vaccine would have larger effect (77). The ideal approach would therefore be a single multistage vaccine that is effective against both acute and latent infection, but no such vaccine currently exists (78, 79). Research in this area is discussed below.

The State of the Art for TB Subunit Vaccines

Subunit vaccines offer significant advantages over BCG. Since they need not be restricted in their growth (or are designed not to require growth in the host) by prior immunity to mycobacteria, their activity in individuals sensitized by environmental mycobacteria or BCG should not be impacted. In a highly cited study, six different atypical mycobacteria strains isolated from soil and sputum samples from Karonga district in Northern Malawi (a region in which BCG vaccination has no effect against pulmonary TB) were investigated in the mouse model. Two of these strains from the *Mycobacterium avium* complex were found to block BCG activity completely. Importantly, the efficacy of a subunit vaccine (in this case the Ag85B-ESAT6 fusion discussed below) was completely unaffected by prior sensitization (28). This makes subunit vaccines highly attractive for the boosting strategy. In addition, most subunit vaccines under development use either replication deficient vectors or are non-living, meaning that they pose no threat even in HIV-positive individuals. This makes them suitable for vaccination programmes in TB-endemic regions, where the TB and HIV epidemics are ever more closely intertwined.

Pre-exposure Subunit Vaccines

The observation that immunization with live mycobacteria induced a higher degree of protection than that with killed bacilli had a major influence on the search for immunologically relevant TB antigens. It led to the hypothesis that proteins secreted by living bacilli in the phagosome are the first antigens to be presented to the immune system in the early phase of infection and

consequently an immune response towards these proteins might be more effective at stimulating a protective immune response (80, 81). In 1991, Andersen and colleagues defined a short term culture filtrate (STCF) enriched in secreted antigens from MTB (82). Several laboratories subsequently reported the protective effect of vaccination with culture-filtrate proteins (CFPs) prepared from log-phase MTB cultures in mice and guinea pigs (10, 83, 84) and demonstrated that the protection was transferable by CD4+ T cells (10). The demonstration that non-living vaccines based on secreted proteins could effectively protect against subsequent MTB infection in animal models led to the initiation of extensive antigen discovery programmes which aimed to identify crucial antigenic molecules in culture filtrates.

One approach to systematically analyse the STCF proteins was to divide the proteins into a number of pools according to molecular mass. This was achieved by separating the proteins into narrow molecular mass regions by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by eluting the proteins using the Whole Gel Eluter. With this method, 15–30 protein fractions were obtained and each pool contained only a few proteins, all in the same molecular mass region. These fractions were used to stimulate cells obtained either from MTB-infected mice, cattle, or human TB patients and the IFN-γ release induced was used as a marker of immunological relevance. Two narrow molecular mass fractions of STCF, containing molecules of low mass (<14 kDa) and medium mass (26-34 kDa), were found to be particularly strongly recognized by cells from infected mice and patients with minimal TB (85, 86). Subsequently several proteins were identified in these fractions and these became the basis for most of the first new vaccines, as discussed below.

ESAT-6

One of the active components in the low molecular mass fraction, a 9.8 kDa protein, was purified and named Early Secreted Antigen Target (ESAT-6). Genomic analysis further demonstrated that ESAT-6 was a member of a multigene family of proteins, the esat-6 gene family, encoding several immunodominant proteins which are strongly recognized by T cells from MTB infected individuals or in animal models of TB. The genes encode proteins of 90–120 amino acid residues and have some degree of sequence similarity (20–35 per cent) to ESAT-6 and are organized in operon-like structures, paired two and two preceded by a pair of genes that encode proteins of the PE and PPE families. The esat-6 gene family consists of 14–23 proteins dependent on the criteria used (87) and can be further divided into subfamilies. One such family consists of the homologous proteins TB10.4 (Rv0288), TB10.3 (Rv3019), and 2.9 (Rv3017c) (87). Another esat-6 subfamily is the Mtb.9.9 family, consisting of five open reading frames (Rv1037c, Rv1198, Rv1793, Rv2346c, and Rv3619c)

(88) and also interestingly, the neighbouring Open Reading Frames (ORFs) (Rv1038, Rv1197, Rv1792, Rv2347c, and Rv3620c) also make a subfamily (the QILSS family). The function of ESAT-6 is not fully known. It is secreted as a heterodimer with another esat family member, CFP10, and the heterodimer has been shown to disrupt planar membranes (89, 91). Extensive vaccine studies have been performed with ESAT-6, which has been found to be an immunodominant target for IFN-γ producing T cells from infected mice (92, 93) and several ESAT-6 epitopes, that are frequently recognized by MTB-infected patients, have been identified (94). As a vaccine, ESAT-6 was shown to induce protective immunity either as a Deoxyribonucleic acid (DNA) vaccine (95, 96) or as a subunit vaccine (97). In some studies, the protective efficacy was even comparable to that of BCG (97).

Ag85

In the molecular mass fraction from 26-34 kDa, the three members of the Ag85 family have received the most attention. The family includes three closely related mycolyl transferases of 30–32 kDa mass (antigen 85A, 85B, and 85C) secreted by MTB. Both Ag85A and Ag85B have been shown to be among the most potent antigen species yet identified—they are major targets of human T cell responses to MTB and leading vaccine candidates (95, 98–103). Ag85B has been shown to induce partial protection in murine models of infection (98, 99). In guinea pigs, vaccination with purified Ag85B protein also induces substantial protective immunity against aerosol challenge with MTB (104) and a recombinant BCG vaccine expressing and secreting the Ag85B protein (rBCG30) induced stronger protective immunity against aerosol challenge with MTB than a conventional BCG vaccine (105). Finally, a vaccine based on recombinant modified Vaccinia virus Ankara expressing Ag85A (MVA85A) was shown to significantly boost BCG-primed and naturally acquired antimycobacterial immunity in humans (106).

Ag85B–ESAT-6

Due to the complexity of the host immune response against TB and the genetic restriction imposed by major histocompatibility complex molecules, it has become clear that an effective subunit vaccine containing multiple epitopes may be required to ensure a broad coverage of a genetically heterogeneous population. Recently, we showed that vaccination with a fusion protein consisting of Ag85B and ESAT6 promoted a strong immune response, which was highly protective against TB in the mouse, guinea pig, and non-human primate models (104, 107, 108). This fusion antigen is also effective if delivered in a viral vector or as a DNA vaccine (109). Importantly, Ag85B–ESAT-6 was more protective in both mouse and guinea pig models than either of the single components (109). The first clinical trial of this

fusion molecule, using IC31 as an adjuvant, and given by intramuscular injection, conducted in Holland, showed the vaccine to be well tolerated and highly immunogenic in humans, with strong responses persisting at nearly a year after vaccination (van Dissel et al., submitted). In humans, as in the animal models, recognition of the hybrid molecule was stronger than that generated against either component. Phase I studies have been successfully completed in Holland and Ethiopia, and the vaccine is currently in a Phase II study in Ethiopia.

However, there is one significant drawback to the Hybrid1 vaccine. The ESAT-6 antigen is a mycobacterial virulence factor found only rarely outside the MTB complex and contained in the first genetic region to be lost from BCG during the attenuation process (it is thus absent in all BCG strains). The fact that it is strongly immunodominant makes it (together with the genetically linked molecule CFP10) an extremely valuable diagnostic reagent, since an immune response to these genes can differentiate infection with MTB from BCG vaccination or exposure to common commensal mycobacteria. These two genes are the basis of the two most successful new commercial diagnostic tests (94, 110–113) and there is the worry that vaccination with Hybrid1 might undermine the specificity of these tests. Although preliminary data indicates that Hybrid1 vaccination in fact generates a relatively weak ESAT-6 response (van Dissel et al., submitted) there remains enough concern that finding a vaccine as effective as Hybrid1, but which does not contain ESAT-6, has been a priority for some years.

TB10.4 and Ag85B–TB10.4

In our search for a replacement for ESAT-6, we focused on TB10.4 (Rv0288). TB10.4 is as strongly recognized as ESAT-6 in TB patients and is also recognized in BCG-vaccinated donors (in contrast to ESAT-6) (87, 114). Vaccination with TB10.4 induced significant protection in the mouse model (115). Fusing Ag85B to TB10.4 produced an even more effective vaccine, which induced protection against TB comparable to both Ag85B–ESAT-6 and BCG and superior to the individual antigen components. Thus, Ag85B–TB10.4 represents another new promising vaccine candidate against TB (115). This vaccine construct (now called Hyvac4), delivered in the same IC31 adjuvant already used successfully for the Hybrid1 trials, has also progressed to clinical trials.

Rv1196–Rv0125

Other vaccine developers have taken a similar strategy. The pharmaceutical company GlaxoSmithKline (GSK) has developed a similar vaccine (M72) which consists of a fusion of two MTB proteins, the PPE family member Rv1196, and the putative serine protease Rv0125. Rv1196 is inserted into the middle of Rv0125, which is thus present as two fragments, to ensure it has no

enzymatic activity. This construct is delivered parenterally in the novel adjuvant formulations AS02A and AS01B, containing a cationic, lipophilic vehicle mixed with a detoxified form of the TLR 4 ligand, monophosphoryl lipid A. First tested in 2004, this vaccine has undergone reformulation due to concerns about adjuvant reactogenicity, but has now completed Phase I/II trials in Europe and the United States and is now in Phase II studies in Africa.

Post-Exposure Subunit Vaccines

As noted in the introduction, the ability of MTB to develop a latent infection allows it to outlast an immune response generated by vaccination early in life. Although there is a possibility that new generation booster vaccines might protect against reactivation of latency, the general consensus is that they will not. Those vaccines in clinical development so far have all been assessed as prophylactic vaccines and the measure of their efficiency has been their ability to restrict early bacterial growth and dissemination. Preliminary studies suggest that they may have limited activity against dormant bacilli.

This is not particularly surprising as MTB is able to establish latency and survive in an intracellular habitat for many years by making major changes in gene expression and, therefore, presumably in the antigenic repertoire presented to the immune system. Until recently, little has been known about the conditions that induce dormancy and the bacterial response to those conditions. It has been known that control of bacterial replication in animal models requires production of IFN-γ, TNF-α, and nitric oxide (116) and that exposure of the bacteria or bacterially infected cells to these agents in vitro or to conditions thought to reflect the conditions inside the granuloma, such as limited access to iron, oxygen, or nutrients, leads to a dramatic down-regulation of genes which are highly recognized in TB patients in the early phase of infection—including well-studied immunodominant antigens such as Ag85 and ESAT-6 (117). Mimicking these conditions and inducing bacterial dormancy in vitro has been the subject of intensive research in recent years. O_2 depletion has been the most comprehensively studied and provides a link between the avascular environment of the encapsulated granuloma and the capacity of MTB to adapt to hypoxic conditions. Wayne et al. demonstrated in a series of important studies that a gradual depletion of O_2 changes bacterial respiration towards nitrate reduction and induces significant metabolic, chromosomal, and structural changes in the bacteria consistent with dormancy (118–120). The first MTB gene to be identified as being induced by hypoxia and potentially involved in latency was hspX (Rv2031c), also known as α-crystallin. More recent work using a whole genome microarray has identified more than 100 genes, whose expression are rapidly altered by defined hypoxic conditions,

and has identified the DosR regulon, which consists of 48 genes that are co-regulated with hspX (18, 121). The DosR regulon is up-regulated by bacterial sensing of low, non-toxic concentrations of NO and appears to prepare MTB for dormancy (19). Similarly, other conditions thought to reflect in vivo infection, such as growth in activated macrophages or within artificial granulomas, have been demonstrated to up-regulate the DosR genes (17, 122). Hypoxia-driven dormancy seems to be reversible as provision of O_2, even after long periods of hypoxia-induced bacteriostasis, results in resuscitation and bacterial replication. Recent data suggest that synchronous resuscitation of surviving dormant bacteria is promoted by pheromone-like substances (the so-called resuscitation promoting factors or Rpfs) secreted from slowly replicating bacteria (123). Some of these substances may also promote bacterial spreading and transmission by dissolving the macrophage cell wall through lysozyme-like activity (124).

Nutrient starvation is another factor expected to be encountered by the bacteria in vivo and therefore has been used in vitro by Duncan and colleagues to induce a state of non-replicating persistence with decreased respiration. Proteome and microarray analysis demonstrated that a large number of transcriptional changes occurred but, interestingly, although some of the DosR genes were also up-regulated by starvation, the overall pattern differed significantly from that induced by hypoxia, which would suggest the involvement of a regulon different from DosR (125). Many of these changes appeared to involve lipid metabolism, consistent with earlier findings that long-term survival in the murine lung requires that MTB express isocitrate lyase, an enzyme essential for the metabolism of fatty acids (126). Importantly, this gene was necessary for replication of the bacteria in the late stage of infection in normal mice, whereas bacteria with a disruption of the gene still multiplied in IFN-γ KO mice. This suggests that the metabolism of MTB in vivo is profoundly influenced by the host response to infection. It is possible that activated macrophages are more easily able to deprive the bacteria of nutrients (perhaps by resisting changes to phagosome trafficking (36, 43)) and that the bacteria switch their metabolism to fatty acid degradation in response to this. This hypothesis is supported by the examination of the transcription profile of MTB grown in activated murine macrophages or in the lungs of infected mice, which indicates that MTB adapts to immune activation by expressing fatty acid–degrading enzymes and secreting siderophores to facilitate the acquisition of iron (17, 127). This finding underscores the complexity of the bacterial transcriptional response to the multiple environmental signals encountered during its intracellular lifestyle and recent work (discussed in the last section of this chapter) is focusing on how to design vaccines that target the bacteria in its latent phase.

Delivery of Subunit Vaccines: Routes and Adjuvants

The currently used adjuvants for human vaccines (based on aluminum salts) are only effective in vaccines that require a humoral response (e.g. diphtheria, tetanus, and hepatitis B vaccines) since they bias the immune response towards the Th2 pole (128). Protection against TB, however, requires a CMI-based response and animal studies have shown that the most effective vaccines generate a Th1 response and that the use of alum-based adjuvants may actually decrease protection against MTB (129).

Many of the leading new adjuvant candidates, although developed independently of each other, have a very similar composition, based on the recognition of these facts. The IC31 adjuvant from Intercell, which is in clinical trials (together with Ag85B–ESAT6 (108) and Ag85B–TB10.4 (115)) is a mixture of oligodeoxynucleotides and polycationic amino acids (130). The AS2 adjuvant developed by GSK (and already in clinical trials with the MTB72f vaccine—a fusion molecule comprised of the two proteins, Rv1196 and Rv0125) consists of an oil-in-water emulsion containing 3-deacylated-monophosphoryl lipid A, a detoxified form of lipid A, and a purified fraction of *Quillaria saponaria*, known as Quil A, mixed with the TLR 4 ligand monophosphoryl lipid A (131, 132). The spontaneous assembly of liposomes (multilamellar vesicles), when polar lipids are mixed in an aqueous environment, has been used to encapsulate antigens or immunomodulators that are present during the process and it has been shown that this can augment the efficiency of vaccination against MTB (133). In particular, cationic liposomes (e.g. the Cationic Adjuvant Formulation (CAF) family of adjuvants from the Statens Serum Institut (SSI)) have been used extensively with TB vaccines and consists of immunostimulants such as monophosphoryl lipid A and trehalose dibehenate in cationic liposomes based on lipoid surfactants (9, 97, 129, 134). All of these adjuvants are strong promoters of Th1 immune responses and both IC31 and cationic liposomes are positively charged vehicles, which are believed to target the antigen/adjuvant complex to negatively charged membrane structures, improving acquisition by phagocytic cells and thereby improving access to both MHC class I and MHC class II processing pathways (130, 135–137).

The adjuvants described above are intended for injection, and the current and proposed clinical trials all use that method. However, the potential for stimulating an immune response at the natural portal for MTB infection, combined with the possibility of 'needle-less vaccination', has led to an interest in mucosal vaccination. The promise of the mucosal route for TB vaccination has already been demonstrated, with levels of protection equivalent to or better than that obtained by subcutaneous vaccination (138, 139). The mucosal route may impose its own specific requirement as to the delivery systems and adjuvants particularly suited to this route may need to be optimized. One

example of this is LTK63, a modified, heat-labile enterotoxin from *E. coli*, which has been tested as a candidate adjuvant, developed by Novartis. It has been evaluated in clinical trials and animal models as a nasal adjuvant and stimulates both mucosal antibody production and a significant IFN-γ response (140, 141, 142). However, concerns over potential toxicity have restricted its further development.

Using Live Vectors other than Mycobacteria to Deliver Mycobacterial Antigens

Viral vectors, such as adenovirus or Vaccinia, trigger a Th1-dominated immune response, characterized by elevated induction of IFN-γ and thereby bias the response to the MTB antigens they express in the same direction (143, 144). It was originally thought also that virally delivered vaccines should also stimulate greater CD8 recognition of the expressed MTB antigens.

The first vaccine of this type to be tested in humans was MVA-85A (106, 139), a recombinant, replication-deficient Vaccinia virus, expressing antigen 85A from MTB. This vaccine has performed well in animal models and results from initial human trials found that it was also highly immunogenic in humans (106, 139). Interestingly, in BCG-vaccinated individuals, even for those who received their BCG vaccination years earlier, the magnitude of the anti-antigen 85A response was even greater than in naïve donors, suggesting that the vaccine was indeed boosting prior immunity (145). Side effects are apparently relatively mild and MVA-85A is now undergoing multiple Phase I/II trials in African populations, where data similar to the initial European trials has been obtained (146).

The second virally vectored vaccine is Aeras-402 Ad35, a replication-deficient (it lacks the E1 and E3 genes, so it can only replicate in cultured cell lines that express these genes) recombinant adenovirus-35 (147) expressing a fusion of antigen 85A, antigen 85B, and TB10.4. All three antigens are present in BCG and all are highly immunogenic in humans. There is, as yet, very little data on the efficacy or safety of this vaccine in animal models (148), but the vaccine has passed initial Phase I testing in the United States and is currently in the second stage of Phase I testing in populations from a TB-endemic area: in this case, South Africa.

Although these vaccines are not restricted by (and may even benefit from) prior sensitization to mycobacteria, they must still face issues of sensitization. In the case of MVA-85A, many adults, especially in TB-endemic areas, will have been vaccinated with the Vaccinia vaccine and there is some data to suggest that this can reduce the efficacy of Vaccinia-vectored vaccines (149). However, it is not known what effect this will have in a clinical situation where the duration between vaccinations will generally be many years. For adenovirus-based vaccines, there is similar evidence that prior humoral responses can

reduce vaccine efficacy, and this has shaped the choice of a type 35 adenovirus as the vector: serological responses to type 35 adenovirus have a relatively low frequency (from 3 to 5 per cent in developed countries to 20 per cent in Africa) compared to type 5 adenovirus. However, as with MVA, the practical effect of this pre-existing immunity remains unknown.

Clinical Considerations: Practical Issues

The Legacy of a Century of Vaccine Trials: BCG and the Koch Phenomenon

Although all of the vaccines currently in clinical trials can, in theory, prime immunity as well as BCG, and there are clear operational and cost benefits to standardizing on a single vaccine protocol, the approach of today's vaccine developers is split by the vaccines they have to hand. The boosting vaccines in development are designed to be given after BCG, so testing in BCG vaccinees makes perfect sense. Recombinant mycobacterial vaccines, on the other hand, are intended as BCG replacements. It is not clear if they will be any more effective as boosters than BCG has been. However, all of the current vaccine developers have chosen to test their vaccines initially in adults, through Phase I and Phase II. There are two reasons. The first is the difficulty in replacing BCG with a new, unproven vaccine, given BCG's clear beneficial effect on mortality in children, when given at birth, and the fact that none of the new vaccines have yet been proven to work in humans (150, 151). Even without this, testing novel vaccines in infants (who are unable to give informed consent) obviously faces higher barriers to approval than testing new vaccines in adults. The second issue is the growing consensus that immunity induced by BCG wanes over a period of a decade or more (1–3). Without evidence that immunity induced by the new vaccines lasts longer than (or even as long as) BCG, it is possible that boosting will still be required; in which case the benefit to be gained from removing BCG is dubious.

Thus, all of the vaccine programmes so far have elected to test initially in adults, with age de-escalation studies to follow if there are no issues of safety. Questions of safety are especially sensitive for TB vaccine development since the first attempt to develop a TB vaccine by Robert Koch in the late nineteenth century (152). Koch understood that an inflammatory response was essential for the control of the pathogen and he used repeated injections of sterile filtrate from MTB cultures as a therapeutic vaccine in already infected TB patients. Alas, the severe inflammatory immune responses induced in some individuals with active disease proved to be fatal. This reaction (now known

as the Koch phenomenon) appears to be due to overproduction of multiple proinflammatory cytokines, with TNF-α (153) most prominent among them. None of the vaccines described above are therapeutic vaccines designed to treat active TB, but their use as adult boosters inevitably means that in TB-endemic regions some individuals who are vaccinated will be latently infected. While there is no reason to expect a reaction similar to the Koch phenomenon with today's booster vaccines (which have been carefully screened for their inability to cause adverse events during development), the mere possibility means that a conservative approach is mandated.

As a result, all of the subunit vaccines in or near clinical trials are progressing through testing in three populations: first in mycobacterially naïve individuals (no identified risk of TB, not BCG-vaccinated, and not positive in the TST or more-specific in vitro diagnostic tests such as the Quantiferon or T.Spot tests); then in mycobacterially sensitized healthy individuals (BCG vaccinated, but not positive by in vitro diagnostic tests such as the Quantiferon or T.Spot tests and without identified risk of TB); and finally in healthy, latently infected individuals—generally former TB patients. The difficulty of identifying latently infected individuals means that the vaccines have all undergone initial testing in Europe or North America, where the possibility of an undiagnosed latent infection is greatly reduced—with clinical trials moving to TB-endemic populations once this first screening is complete.

The Effect of HIV

HIV and TB are a particularly difficult combination with each apparently worsening the other (8, 23, 24). HIV by itself was not originally expected to be a significant problem. Although BCG vaccination is recommended against in individuals who are already HIV-positive, BCG is used widely in areas where HIV-positivity is also high and the risk of developing a disseminated BCG infection from a prior vaccination appears to be very low in HIV-positive adults. In addition, the new vaccines are being developed with HIV in mind: the recombinant BCG strains are less virulent in immunodeficient animals than the parental strains (the attenuated MTB strains are apparently unable to persist in vivo); the two viral vectors are replication-deficient and the recombinant proteins are not expected to have HIV-related issues. Nonetheless, given the likelihood that the vaccines will be administered to HIV-positive recipients at some point if taken into use, all groups have elected to specifically test safety in HIV-positive adults in early clinical trials.

A more profound effect of HIV is likely to result from observations that suggest that BCG is much more likely to cause disseminated disease in infants who are vaccinated while already HIV-positive and that mortality is very high in these cases (154, 155). Since testing for HIV in infants is notoriously

unreliable (and is also resource-consuming) this raises serious issues. Should BCG be deferred on suspicion of HIV infection—and accept the risk that a vaccine delayed is often a vaccine not actually given? Or should we vaccinate and accept elevated mortality among HIV-positive infants? As yet there is no good answer, but the alteration in risk/benefit analyses of BCG vaccination is starting to raise interest in the prospect of replacing BCG.

Phase II Trial Issues: Finalizing Vaccination Protocols

The most advanced vaccines are now entering Phase II studies. While the emphasis in Phase I has been on safety, in Phase II questions of efficacy and target population start to become more important. With regard to the latter, age de-escalation to adolescents (for boosting vaccines) or to infants (for priming vaccines) are the next step. Where infants are targeted, interference with existing scheduled vaccines needs to be addressed. And all vaccines need to finalize the question of the vaccination schedule to be used (how many doses and when they will be given). The latter question is particularly relevant for non-replicating vaccines such as adjuvanted proteins: such vaccines have generally required multiple (two or three) doses to achieve maximum immunogenicity and the timing between vaccinations also has an effect on the outcome. In addition, for booster vaccines, there is the question about the time interval after BCG. The optimal period between BCG vaccination—normally given to infants—and boosting is unknown. The only data available in humans comes from MVA-85A studies, where it appears that boosting can be effective even after decades (145, 146, 156); if corroborated and applicable to other vaccines, this would suggest the timing of a booster vaccination relative to the age of initial MTB infection in the target population is a more important variable than the time from BCG vaccination.

Assessing Vaccine Efficacy

The most vexing question to be addressed in Phase II trials is that of efficacy. Clinical end points (the amount of TB in a vaccinated versus a non-vaccinated population) are the true measure of efficacy, but these are really only obtainable in Phase III studies. Unfortunately, the expected high cost of Phase III trials means that a vaccine which cannot demonstrate some efficacy in humans is going to find it hard to secure the resources needed to progress into a Phase III trial. Researchers have therefore been looking for immunological markers to measure the effect of the vaccines. While not, technically, a requirement (vaccines such as those for rotavirus or papillomavirus were developed without immunological markers of efficacy), it would certainly help the progress of a new vaccine to Phase III trials if some evidence of efficacy could be found. Regrettably, the TB field still lacks a clear marker of vaccine efficacy. The

clear importance of IFN-γ for the control of infection has meant that all of the studies to date have focused on the ability of a vaccine to stimulate IFN-γ production (primarily by ELISA, ELISpot or both, though GSK and Aeras have also incorporated Fluorescence-activated cell sorting (FACS) analysis into their trials and several groups have collected plasma for serological analyses). In Phase I, IFN-γ production has been used to show that the vaccine is immunogenic and generates specific antigen responses in human recipients. Given the extensive prior screening in animal models—and the fact that the vaccine antigens used were originally selected on the basis of recognition in humans—it is no surprise that all of the vaccines tested so far are strong inducers of antigen-specific IFN-γ in humans. But is measuring the magnitude of IFN-γ production enough?

The general consensus in the TB field is that it is not. While IFN-γ production to antigens of the pathogen is essential for protection, the 'quality' of the response is also important and if the immune response is focused on the wrong target, you can generate a strong, antigen-specific IFN-γ response—without inducing protection (157). What is needed is a better marker, a correlate or surrogate of immunity, or at worst, of infection. Searching for correlates of immunity for TB is a very active field, and beyond the scope of this chapter, but the questions of correlates and surrogates of immunity is discussed in detail in a recent review (158).

In the absence of a defined correlate (at least so far), a possible alternative is the use of a proxy marker of infection. Large clinical vaccine trials are required for TB vaccines because most MTB infections do not present immediately as clinical cases, leading instead to latent infection (LTBI). The clinical end point is thus the difference in this small number of cases in the vaccinated and unvaccinated groups. If it were possible to use the rate of infection rather than disease as an end point, the number of participants required for a study would fall dramatically (in a highly TB-endemic setting such as the Western Cape, it may be possible to do a trial with as few as 2,000 adolescents). It remains unknown if this is possible, but a small recent study of BCG vaccination in Turkey suggests that vaccination can in fact prevent the establishment of infection (159, 160) (or perhaps more probably, reduce the infection to a level where it cannot be detected by immunodiagnosis). Given that recent studies suggest that the magnitude of the anti-ESAT-6 response may reflect the severity of infection (161, 162) and may predict later breakdown with disease (46, 163–165) and that ESAT-6 conversion in large field studies of adolescents is on the order of 10 per cent per year (W Hanekom, personal communication), measuring the conversion to antigen-positivity by immunodiagnosis rather than the rate of subsequent breakdown may offer the possibility to greatly reduce the size and cost of Phase III studies. In the meantime, work is going

ahead on establishing sites in multiple countries capable of carrying out the very large studies required for Phase III trials.

Phase III Trial Issues

Phase III trials are intended to demonstrate that a product is efficacious. However, given that BCG appears to be efficacious against TB in infants and children (5, 25, 151, 160), and that the source of most new cases is adult pulmonary TB, the real prize for a new TB vaccine is efficacy against adult disease. Previous TB vaccine trials (with BCG) measured efficacy using a simple comparison of the number of TB cases in the vaccine arm compared to the placebo or unvaccinated arm. If using a vaccine given at birth, like BCG, this is manageable in trials of infant TB. But when looking at disease in adults, the clinical end point may be 15–25 years after vaccination; in other words, after the initiation of the trial. Combine this with the low incidence of TB disease—typically much less than 1 per cent, even in TB-endemic regions (7)—the fact that exposure to TB is not always easy to define and that the disease can take up to decades to develop, and to obtain reliable data, earlier studies have required decades of observation of huge numbers of participants (sometimes in excess of 100,000) (166). There is little appetite for such studies today. Fortunately, boosting vaccines for adolescents are given closer to the peak years for adult TB and so studies can be both shorter and smaller than those of the past. Nonetheless, it is still estimated (167) that the time for such a trial for a boosting vaccine would be 5–10 years and require approximately 40,000 participants for a vaccine that is 70 per cent efficacious. This is far from trivial, contributing to the pressure to set selection criteria for vaccines before starting a Phase III trial.

One approach traditionally used for diseases with a low general incidence is to target an at-risk population with a relatively elevated incidence of the disease in question. Adult HIV+ individuals in TB-endemic areas would fit this definition, as TB in these individuals is both frequent and rapidly progressive. But their immunodeficiency makes their selection for this role doubtful. The other approach is to use infection, rather than disease, as a clinical end point. This is extremely appealing but, as discussed above, no consensus exists on whether this is feasible, and we are unlikely to get conclusive data within the next few years. Forward planning at the field sites, then, is based on the assumption that very large trials may be needed.

Things are a little easier for vaccines designed as direct replacements for BCG. These can be done as a comparison of the new vaccine to standard BCG vaccination (it would be unethical to include a placebo arm, given the evidence for BCG efficacy (150)). Recent analyses suggest such a study could be smaller than the adolescent group discussed above (perhaps as few as 6,000), due to the susceptibility of infants to TB, and the rapidity with which disease

develops in infected infants means that it could be completed in only two to three years. This is a much more attractive approach, reducing both the length of the trials and of course its costand, crucially, reducing the time to develop a registered product. As a result, even boosting vaccines—which in animal studies have been able to show that boosting BCG could produce better control of infection than BCG alone over quite short time frames—may take the route of infant studies as a way of establishing proof of principle. We already know that a subunit vaccine can boost BCG-induced immune responses even after many years (145). If a new vaccine could show improved efficacy during the first few years of life, that is, during the period when BCG immunity is at its most effective, that would be a very strong argument for the utility of boosting in later life, when BCG efficacy appears to wane. Thus, even booster vaccines may be tested first against paediatric TB simply to demonstrate that they are effective in humans, with the effect on adult disease being evaluated as a Phase IV study of the same population. Such studies will require solid data from Phase II trials supporting potential efficacy and age de-escalation studies, but that data should be available within the next one to two years.

Production, Delivery, and Cost

The discussion above has focused on the clinical aspects of vaccine testing, but like any pharmaceutical product vaccines face practical constraints and in some ways these are even more stringent for a potential TB vaccine. The product characteristics which are most important include cost and ease of manufacturing, product purity and stability, and the use of the product under field conditions. TB vaccines are most needed in some of the world's poorest countries, which means that cost is a significant issue. In addition, such countries generally have a poor health infrastructure. Limited cold chains and poor documentation place a premium on a vaccine's stability and shelf life at room temperature. While it is possible to enter Phase I trials with an experimental product, Phase II has stricter requirements and the product tested needs to be closer to, if not identical with, that which will be taken into Phase III trials. This means that in addition to clinical data, product characteristics will be weighted when it comes to selecting the first candidates for Phase III trials. It is impossible at this stage to make any firm predictions—no Phase II data is yet available for any of the vaccines—but the demonstration that the Hybrid1 vaccine and its adjuvant can be freeze-dried to give a product with a very long shelflife (years, rather than months) is likely to boost the fortunes of recombinant protein vaccines. Both recombinant BCG and the recombinant protein vaccines being tested can be produced in bulk at low cost, so that the cost hurdle does not seem to be insuperable for them. It remains to be seen how non-mycobacterial vectors will fare in these areas.

Beyond Phase III: Future TB Subunit Vaccines?

The vaccines currently in clinical trials, and those closest to clinical trials, are the first new TB vaccines in over half a century. Bringing them to this stage has involved major advances in virtually every facet of vaccine development. However, even if they successfully induce immune responses effective against MTB, it is not certain that they will solve the TB problem. MTB is a particularly difficult pathogen to control for many reasons, but prime among them is its ability to persist for decades in a latent state. This means that the eradication strategy used against smallpox and polio, where the vulnerable population in a limited geographic area was protected by vaccination and the pathogen eliminated in that region, is not really viable. In every area of the world, latent TB remains—able to infect susceptible individuals whenever it reactivates. Moreover, it is not clear if any vaccine can generate lifelong immunity, so even vaccinated individuals may become susceptible again in time. This means that vaccination efforts must be continuous—a difficult task in the world's poorest nations. As a result researchers are now looking for ways to tackle the problem of latent TB infections.

The current 'first generation' new vaccines are all prophylactic vaccines, designed to be given prior to infection to prevent disease. Evidence from animal models suggests that even though these new vaccines represent improvements over BCG for this purpose, none of them guarantee sterilizing immunity (76, 107, 109). In humans, BCG does not prevent the establishment of latent TB infection as clearly demonstrated by the immense numbers of latently infected individuals within the BCG vaccinated population worldwide. Similarly, in animal models, even those vaccines which increase survival of infected animals lead to control of bacterial growth at a lower level than in unvaccinated animals; basically, these vaccines seem to prevent acute disease, but we have no evidence that they prevent the establishment of latency.

The rarity of disseminated BCG disease in HIV-infected adults who received BCG vaccination well in the past suggests that, unlike MTB, BCG is not able to establish latent infections. It is therefore possible that part of the attenuation of BCG is an impairment of its dynamic response to a changing environment and transition to a latent state. This is supported by recent data indicating that BCG is unable to stimulate strong responses to antigens which are commonly recognized by humans latently infected with MTB (168). Obviously, this would also imply that BCG may induce only limited immune response to some of these latency associated antigens and this may be one of the reasons for the failure of BCG in preventing latent TB. If so, it may also be true of the first generation vaccines under development which have focused on expression of antigens expressed by actively replicating MTB.

The goal for second generation, so-called multistage, vaccines is that they should target the latent stage of disease more efficiently than BCG and also prevent disease from acute infection. While it is possible that post-exposure vaccination will be analogous to boosting BCG, data from animal models suggest otherwise: it may be that vaccines against latent TB need a different panel of antigens than those that have emerged from the last two decades of research, which has focused on preventing disease in primary infections. So far there has been no consistent success in attempts to use these prophylactic vaccine candidates as post-exposure or therapeutic vaccines (169–173). And while there have been some studies indicating therapeutic potential for new TB vaccines (174–176), others have seen no effect against reactivation (170, 177). The reason for these conflicting results is unclear, but it may reflect the quality of the immune response. Most latently infected individuals already have very strong T cell responses to multiple mycobacterial antigens and the requirements for a vaccine to further boost this response is not completely understood.

As a result, efforts have been stepped up to answer two crucial questions. First, what are the immune responses characteristic of containment of latent infection? The rapid reactivation of disease in immunocompromised individuals (e.g. those developing AIDS, or those treated with anti-TNF-α therapy (21–24) indicates that an ongoing immune response is required to control latent MTB infection (178). If we can characterize this immune response, we can design vaccines to foster it. Secondly, what targets is this immune response directed against? The failure of prophylactic vaccines against latent infection in animal models suggests that while memory T cells specific for early antigens may persist after the initial stage of infection, these cells may not contribute significantly to the containment of bacteria during dormancy or resuscitation, perhaps because in these disease phases the bacteria expresses a different panel of antigens—a hypothesis supported by human studies. The consensus from these studies is that antigens such as ESAT-6 or Ag85, expressed during invasion and early growth of the pathogen, are strongly recognized in patients with active disease (86, 94, 179). However, as the bacteria react to the host's immune responses and the environment in the developing granuloma, there is expression of a novel repertoire of antigens and responses to these antigens signal controlled infection. Multiple studies in humans from TB-endemic and non-endemic regions support this notion. Differential analysis showed that latently infected individuals responded to more latency antigens with stronger IFN-γ responses than did TB patients, while the opposite profile was found for the early antigen CFP10 (part of the ESAT antigen family). In particular, Rv1733c, Rv2029c, Rv2627c, and Rv2628 encoded proteins that induced strong IFN-γ responses in latentlyinfected individuals, with 61 per cent, 61 per cent, 52 per cent, and 35 per cent responders, respectively. In a similar study, responses to ESAT-6 were

compared with those to HspX and it was observed that whereas the TB patient group had responses strongly biased towards ESAT-6, the latently infected group had a much more balanced response with substantial responses directed to HspX (180). The relatively low T cell response to HspX in TB patients is in agreement with earlier observations from several laboratories (181).

Common targets for prototype multistage vaccines are therefore those antigens thought to be induced by the conditions encountered by the bacteria in vivo: low oxygen tension, nutrient starvation, and toxic molecules such as nitrogen and oxygen radicals (18, 19, 121, 122, 125). Some of the most actively investigated factors are antigens of the DosR regulon, the majority of which are expressed and recognized by the host immune system during natural infection (182) or the *rpf* genes thought to be involved in bacterial reactivation (182, 183). Recent studies with the Rpf-like proteins have found them to be strongly immunogenic and capable of inducing significant protection against a high dose challenge with MTB (183). Similarly, RV3407, identified by comparing proteomic profiles of MTB and BCG, is an Rpf-like gene product and, when administered as a DNA vaccine, gave significant levels of protection against aerosol challenge (184). While these studies have not yet demonstrated their ability to contain MTB in its latent stage or prevent reactivation, preliminary studies in the mouse Cornell model using antigens induced by starvation of MTB suggest that they can reduce reactivation of the bacteria (author's unpublished data). Taken together these findings indicate that antigen recognition in the different stages of infection is a dynamic process. A change in the bacterial transcriptome is mirrored by a subsequent change in the specificity of the host response and this balance is most likely instrumental in maintaining immune control, containment, and the prevention of reactivation. Much work remains to be done, but the addition of antigens expressed by MTB during latency or reactivation to the well-characterized prophylactic vaccines under clinical testing today may provide the basis for a future generation of multistage TB vaccines with activity against all stages of infection.

Conclusion

While we still have a lot of work ahead of us, we also have reason to be optimistic. At the end of the twentieth century, several research agencies laid out their priorities on TB vaccines (185) and the consensus then was that clinical trials were still some years distant. In fact, the first new vaccines entered clinical trials within two years and in the subsequent few years, more candidate vaccines followed. New adjuvants, effective at stimulating cell-mediated responses and apparently safe in humans, are also in trials. Phase II trials are already

underway with two vaccines and at least two more are expected to reach that stage over the next year. At the same time, more advanced vaccines, which show activity against the latent form of the disease in animal models, are already in late preclinical stages and are entering primate studies, usually the last hurdle before entering the clinical pathway. For the first time ever, we have a TB vaccine pipeline: a process stretching from basic research in TB immunology through laboratory vaccine development to GMP production facilities and the establishment of Phase III trial sites. This pipeline is taking in multiple vaccines, employing many different technologies from numerous research groups—enabling us to progress faster than the first candidates could. We have even reached the point where, for the first time, some vaccines are dropping out of development, not because they do not work, but because even more effective candidates have been developed. In the past, TB researchers have looked for a single cure: BCG was 'the' vaccine, streptomycin was 'the' drug, that would banish TB for ever. Single tools like this, while preventing millions of deaths, have proved to offer only a partial solution. In this regard, the multiplicity of approaches being tested offers comfort. While we cannot say which, if any, of the vaccines currently being tested will be effective in humans in the long term, the fact that so many look promising means that our fortunes are not tied to one particular technology. We can truly say that in the first decade of the twenty-first century, TB vaccine development has come of age.

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