Improving vaccines against tuberculosis

WARWICK J BRITTON¹,² and UMAIMAINTHAN PALENDIRA¹

¹Centenary Institute of Cancer Medicine and Cell Biology, Newtown and ²Department of Medicine, University of Sydney, New South Wales, Australia

Summary  Tuberculosis remains a major cause of mortality and physical and economic deprivation worldwide. There have been significant recent advances in our understanding of the Mycobacterium tuberculosis genome, mycobacterial genetics and the host determinants of protective immunity. Nevertheless, the challenge is to harness this information to develop a more effective vaccine than BCG, the attenuated strain of Mycobacterium bovis derived by Calmette and Guérin nearly 90 years ago. Some of the limitations of BCG include the waning of the protective immunity with time, reduced effectiveness against pulmonary tuberculosis compared to disseminated disease, and the problems of a live vaccine in immuno-compromised subjects. Two broad approaches to vaccine development are being pursued. New live vaccines include either attenuated strains of Mycobacterium tuberculosis produced by random mutagenesis or targeted deletion of putative virulence factors, or by genetic manipulation of BCG to express new antigens or cytokines. The second approach utilizes non-viable subunit vaccines to deliver immunodominant mycobacterial antigens. Both protein and DNA vaccines induce partial protection against experimental tuberculosis infection in mice, however, their efficacy has generally been equivalent to or less than that of BCG. The comparative effects of cytokine adjuvants and vaccines targeting antigen presenting cells on enhancing protection will be discussed. Coimmunization with plasmid interleukin-12 and a DNA vaccine expressing Antigen 85B, a major secreted protein, was as protective as BCG. The combination of priming with DNA-85B and boosting with BCG was superior to BCG alone. Therefore it is possible to achieve a greater level of protection against tuberculosis than with BCG, and this highlights the potential for new tuberculosis vaccines in humans.

Keywords: DNA immunization, Mycobacterium tuberculosis, prime-boost, subunit vaccines.

Introduction  Tuberculosis (TB) is the single most important bacterial infection worldwide. Currently a third of the world’s population is infected with Mycobacterium tuberculosis and annually there are 9 million new cases of clinical TB resulting in 2–3 million deaths. Active infection is present in some 60 million persons at any one time.¹ It is estimated that in the period 2000–20 another billion people will be infected with M. tuberculosis; 200 million will develop TB and 35 million will die from the disease. This huge clinical load is a burden to struggling health services and has an enormous socio-economic impact on a community as the disease affects individuals in the 20–45 age group during their peak period of productivity. The increasing incidence of TB is fuelled by the HIV/AIDS pandemic, as HIV coinfection is the strongest risk factor for progression from infection with M. tuberculosis to active disease.² In immunocompetent individuals infected with M. tuberculosis the lifelong risk of developing clinical TB was previously estimated to be 5–10%, and recent studies in Sydney have confirmed this level of risk. By contrast, subjects coinfected with HIV and M. tuberculosis have an annual risk of 10% for developing clinical TB. Additional factors contributing to the epidemic are the emergence of multidrug-resistant strains,³ including the widely distributed ‘Beijing’ strain, malnutrition and other infections, particularly measles, and socio-political disruption to health services.

Control measures against this intracellular pathogen include effective chemotherapy, improved social conditions to reduce transmission and immunization. Direct observed therapy, short course (DOTS) has been widely implemented over the last 5 years with resulting increases in the cure rate of sputum smear positive pulmonary TB to 70–80% and documented falls in the mortality from TB in many countries. But a major concern raised at the recent World TB Congress was that the case detection rate of new cases of pulmonary TB is reaching a plateau at 40% of the predicted rate in many countries and is unlikely to reach the projected target of 70% by 2013.⁴ This has profound implications for the control of TB worldwide, as this level of drug treatment would fail to control the epidemic.

The only registered vaccine against TB, Mycobacterium bovis (Bacille Calmette-Guérin) (BCG), was first introduced in 1921 and has been widely utilized; however, its effectiveness remains controversial. Meta-analysis of the multiple trials with BCG has concluded that while BCG confers about 80% protective efficacy against disseminated TB, its efficacy against the highly contagious pulmonary disease ranges from 0 to 80%, with a median of 50%. Its use has resulted in a reduction in overall mortality from TB of about 70%.⁵ Its effectiveness in reducing both mortality and long term sequelae from disseminated meningeal and miliary TB in

Correspondence: Professor WJ Britton, Centenary Institute of Cancer Medicine and Cell Biology, Locked Bag No. 6, Newtown, NSW 2042, Australia. Email: wbritton@medicine.usyd.edu.au

Received 22 October 2002; accepted 29 October 2002.
children has justified its use in the Expanded Program of Immunization as a neonatal vaccine. Moreover, recent African studies suggest that BCG immunization is associated with a reduction in mortality from other infectious diseases. Nevertheless, because BCG is less effective at preventing late reactivation and pulmonary TB, BCG immunization has not contributed to controlling the spread of TB. This report will review the current status of efforts over the last decade to develop a more effective vaccine than BCG and highlight the challenges to applying the candidate vaccines in humans.

**Tuberculosis as a target for immunization**

There are a number of significant fundamental problems to be faced in developing vaccines with enhanced protective efficacy against TB. First, infection with *M. tuberculosis* itself or other mycobacteria does not induce sterilizing immunity against reinfection with the same mycobacterium after clearance of the original infection with antibiotics. This is evident following experimental tuberculosis in both mice and guinea pigs; however, host immunity does reduce mycobacterial replication during the second infection and significantly reduces the immunopathology. More recently the advent of molecular fingerprinting of individual strains of *M. tuberculosis* has revealed that exogenous reinfection with different strains can occur in humans. This was first documented in HIV-coinfected subjects, but has been demonstrated in patients without apparent immunodeficiency. Therefore no vaccine is likely to result in sterilizing immunity against *M. tuberculosis* infection.

Second, the lung is the portal of entry of *M. tuberculosis* in most human infection and this provides advantages to this slowly replicating pathogen. The infection is established in alveolar macrophages of the distal alveoli, and has firmly taken root before it is recognized by the adaptive immune response 5–6 weeks later. Although specific lymphocytes, including γδ T cells and CD4+CD8+ αβ T cells are induced during human and murine TB infection, but their relative contribution to protection is less clear. Subjects deficient in receptors for IFN-γ and IL-12 are profoundly susceptible to mycobacterial infections, confirming the absolute requirement of Th1-like T cells for host immunity. Although T cells are essential for protective immunity against *M. tuberculosis*, they are not sufficient and must be brought into close apposition with infected macrophages in granulomas to stimulate bactericidal mechanisms. This formation and maintenance of this granulomatous microenvironment is dependent on other cytokines, particularly TNF-α and lymphotoxin-α (*LTα*).

A fourth factor which may explain some of the variability of the response to BCG is the inherent genetic variation in the immune response to mycobacteria. In addition to the rare mendelian inherited deficiencies of IFN-γ and IL-12 signaling, the human cellular response to mycobacteria is subject to polygenic influences which are currently being unravelled. These include a number of genes influencing macrophage function, such as the genes for nRAMP and the vitamin D3 receptor, as well as the influence of the HLA and other loci. The immune responses to some mycobacterial antigens are also under genetic influence. These genetic effects may well influence both the induction of T cells and the expression of antitymbacterial effector mechanisms following BCG or any future anti-TB vaccines.

Any antimycobacterial vaccine could be used in subjects pre-exposure, to prevent infection, postexposure to prevent the development of disease or as an immuno-therapeutic to act with antimicrobials to increase the rate of clearance of mycobacteria. Some past BCG trials failed to distinguish between these two aims and included both naïve and *M. tuberculosis*-infected subjects, so confounding the results of their analysis. Clarifying the selection of patients will be important in future trials. Pre-exposure and postexposure vaccines are predicted to have different effects on the incidence of active TB within a community. Given the large number of subjects already infected with *M. tuberculosis* who are expected to develop clinical TB, any vaccine which prevented the development of TB in those with established infection would be of substantial benefit. It has been estimated that an anti-TB vaccine with 50% efficacy when administered postexposure would substantially reduce the incidence of TB in a highly endemic community over 20 years, and at a faster rate than a pre-exposure vaccine with equal efficacy.

Modelling the possible effects of anti-TB vaccines has highlighted the independent effects that immunization and
effective chemotherapy have on reducing the transmission of *M. tuberculosis* and the synergistic effects of their interaction.\textsuperscript{19} A pre-exposure vaccine with 50% efficacy at preventing progression to disease when combined with an effective antibiotic treatment for 80% of active TB patients would halve the incidence of TB over 20 years in a highly endemic region.

**What have we learnt from BCG?**

The studies on BCG vaccine efficacy have been extensively reviewed elsewhere,\textsuperscript{20} but some important generalizations can be drawn. First, the effectiveness of BCG against TB in some populations indicates that clinically significant protection can be achieved. Fine has emphasized that understanding the reasons for the wide differences in protective efficacy between populations will provide important insights into how future anti-TB vaccines should be utilized. Second, the protective efficacy of BCG varies in different organs. This may be due to differences in the efficiency of effector mechanisms between the more susceptible lung and other organs, to the size of the mycobacterial load in different tissues or to the time required for boosting of antimycobacterial immunity following infection with *M. tuberculosis*. This emphasizes that vaccine-induced T cells must rapidly expand and be recruited to the lung after exposure to *M. tuberculosis* in order to limit the infection. Third, immunity following BCG wanes with time, and protection in the UK BCG trials was lost after 10 years.\textsuperscript{20} Therefore mechanisms for expanding, maintaining and boosting specific memory T lymphocytes, particularly in the CD4 T cell compartment, are crucial for more effective TB vaccines.

A variety of reasons have been proposed to explain the differences in protection induced by BCG in the multiple trials.\textsuperscript{20} The latitude of the trial site was one factor identified in the meta-analysis to influence the level of protection measured,\textsuperscript{7} and this effect has been attributed to the level of exposure to environmental mycobacteria in the different regions. It is proposed that sensitization to non-tuberculous mycobacteria (NTM) may reduce the apparent efficacy of BCG against TB, either by inducing partial protection and so masking the protective effect of BCG, or by stimulating cellular immunity to antigens shared by environmental mycobacteria and BCG. Such preexisting immunity may inhibit the growth of BCG and the induction of ‘protective’ immunity to antigens shared by BCG and *M. tuberculosis*. Recent experimental infection in mice and cattle has confirmed that prior sensitization to *M. avium* or other environmental mycobacteria does reduce the protective effect of BCG against *M. tuberculosis* or *M. bovis*, respectively.\textsuperscript{21,22} This prior sensitization to some mycobacterial antigens also influences the interpretation of both skin tests and IFN-\(\gamma\) responses postimmunization.\textsuperscript{23} In the mouse model, however, sensitization to *M. avium* did not prevent the induction of T cell responses to an *M. tuberculosis*-specific protein, such as ESAT-6, when delivered as a subunit vaccine. This indicates that although environmental exposure to mycobacteria is a substantial issue in analysing the effect of anti-TB vaccines, it is not an absolute barrier to stimulating protective immunity.

**Approaches to antituberculosis vaccines**

There has been a flurry of major advances in the last 5 years, which are important for the development of new vaccines. First, the genome of *M. tuberculosis* has been sequenced\textsuperscript{24} followed by those for other mycobacteria. The genetic deletions from the sequence of *M. bovis*, which occurred during the initial and subsequent attenuation to BCG, have been identified. These encode a number of highly immunogenic secreted proteins of *M. tuberculosis* and *M. bovis*, including ESAT-6 and CFP10.\textsuperscript{25} Rapid advances in mycobacterial genetics have led to the identification of virulence factors, which enable the intracellular survival of mycobacteria in the otherwise hostile environment of the macrophage.\textsuperscript{26} Attenuated strains of *M. tuberculosis* have been produced either by random mutagenesis or targeted homologous recombination, resulting in the identification of defined auxotrophs of *M. tuberculosis* or *M. bovis*. The original vaccine strain BCG has also been manipulated to produce recombinant BCG (rBCG) strains expressing mycobacterial and foreign genes, either from other pathogens or host immuno-regulatory molecules. Advantages of these live vaccines include the capacity to replicate in the host and the inherent adjuvant properties of mycobacterial cell wall, which may stimulate more prolonged memory T cell responses. Moreover they present an extensive repertoire of antigens which may stimulate CD4 and CD8 T cells and induce protective immunity. The disadvantages include their potential as viable vaccines for uncontrolled replication in immunodeficient hosts, particularly HIV-infected subjects, possible genetic instability, and the possible tissue damage from the vaccines. The latter limits the use of live vaccines for repeat boosting of memory T cell responses. In addition there will be major regulatory problems in obtaining approval for use of attenuated *M. tuberculosis* strains in humans. Therefore any new live anti-TB vaccine will need to have greater protective efficacy than BCG to justify its use in humans.

The second area of development is in subunit vaccines. The availability of the genome sequence has enabled the further characterization of the mycobacterial proteins, which stimulate T cell responses in humans or mice during *M. tuberculosis* infection. A number of these immunodominant antigens are recognized by infected subjects from different ethnic backgrounds and are candidates for inclusion as subunit vaccines. Both CD4 and CD8 T cell epitopes have been identified on these proteins and these may be utilized to assess host response to the vaccines incorporating the antigens. Advantages of subunit vaccines delivered as protein or DNA or in non-replicating viral vectors include their safety profile and the lack of tissue damage or scarring. This permits their repeated use as booster doses to maintain effective T cell memory. Other important factors, however, include the extent of the T cell repertoire required to protect individuals from diverse human populations against TB and a universally effective delivery vehicle which will stimulate the necessary broad spectrum of CD4 and CD8 T cell responses to the limited number of selected antigens. Non-viable vaccines may be more acceptable than BCG in certain circumstances, and therefore candidate non-replicating subunit vaccines, which have equivalent effect to BCG, may be suitable for clinical trials in humans.
Third, the models for preclinical testing of vaccines have been refined. In addition to the murine infection model, which is appropriate for screening candidate vaccines, highly susceptible guinea pigs have been used to determine if vaccines protect against fatal infection and the type of caseating immuno-pathology seen in human infection. Large animal models including primates and cattle have also been developed, and the former may be to confirm efficacy before human trials.

**Live mycobacterial vaccines**

Table 1 summarizes the results of protection studies with defined auxotrophs of *M. tuberculosis* and rBCG. The purC strain was the first tested and found to induce skin test reactivity and protection in a small number of guinea pigs. Subsequently a number of amino acid auxotrophs have been observed to induce partial protection in mice challenged with *M. tuberculosis*. A major issue in the practical application of attenuated organisms is their long-term stability when processed through animals or humans. Therefore Jacobs and colleagues have produced a stable strain by targeted disruption of two genes in the synthetic pathway for panthenic acid. This panCD auxotroph was markedly attenuated and survived for prolonged periods in immunodeficient mice, when compared to BCG-infected animals. Mice immunized with this strain were protected against *M. tuberculosis* infection to a similar degree to BCG, and showed a reduction in pathological damage to the lung. Such strains will require extensive testing in other models of infection to confirm their stability.

A different approach is to improve the effectiveness of BCG which has been proven a safe vaccine in the vast majority of vaccinees. Recombinant BCG have been developed expressing bacterial and viral antigens and shown to induce potent T cell responses and a variable degree of protection dependent on the pathogen tested. This technique has been used to express either *M. tuberculosis*-specific proteins in BCG or increased quantities of major shared antigens. For example, the gene for antigen 85B, which is widely recognized by *M. tuberculosis*-infected subjects, was expressed in BCG under the control of the powerful Hsp65 promoter. Guinea pigs immunized with this rBCG-85B showed reduced immunopathology and increased survival following TB challenge. Expression of ESAT-6 in BCG leads to the induction of strong T cell responses to this protein (Triccas, unpubl. obs) and the protective efficacy of this strain is being assessed. A variant on this theme employed the gene for listeriolysin (LO) which is responsible for enabling *Listeria monocytogenes* to escape from the phago-lysosome into the cytoplasm of infected cells. The BCG expressing LO also enters the cytoplasm of infected macrophages and this was associated with the induction of more potent CD8 T cell responses in infected mice, as the mycobacterial peptides were processed through the proteosome into the MHC Class I pathway.

Another development has been the expression of mammalian cytokines in rBCG. Somewhat surprisingly the eukaryotic proteins are secreted from the slow growing mycobacteria and despite the differences in post-translational modifications between mycobacteria and mammals, functional cytokines are released. The initial studies with IFN-γ-secreting BCG demonstrated increased immunogenicity in immunized mice. More recently rBCG expressing the immuno-regulatory cytokine IL-18 has been found to induce more potent IFN-γ-secreting T cell responses to mycobacterial proteins (Triccas, unpubl. obs). Alternatively, the addition of the cytokine antagonist, latency activating peptide which inhibits the activity of transforming growth factor-β, was found to increase antimycobacterial responses.

**Subunit vaccines against tuberculosis**

The demonstration that immunization with culture filtrate proteins of *M. tuberculosis* partially protected mice against TB raised the possibility of effective subunit vaccines. Proteins secreted by *M. tuberculosis* early during growth are potent T cell antigens in infected humans and mice. There are over 100 proteins in the early culture filtrates but some components are apparently immunodominant. These include the Antigen 85 complex (Ag85), which is a family of three closely related mycolyl transferases with homologues in all mycobacterial species, and proteins restricted to *M. tuberculosis* complex, such as ESAT-6. Up to 14 RD regions were deleted during the attenuation of *M. bovis* into the vaccine strain BCG, and the RD1 region, which encodes ESAT-6, was the first deleted. Immunization of mice with Ag85B, Ag85A or ESAT-6 with a variety of adjuvants partially restricted...
growth of *M. tuberculosis* after aerosol challenge (Table 2). More recently a fusion protein of Ag85B and ESAT-6 was shown to be more effective than either alone, possibly because of the increased stability of ESAT-6 epitopes. A number of other secreted proteins are encoded by the 129 open reading from (ORF) which have been deleted from BCG, and some of these may also be vaccine candidates.

An alternative approach has been to define the genes encoding proteins recognized by human T cells by expression cloning. A number of these proteins have been found to induce partial protection in mouse and guinea pig challenge models and are currently being evaluated in primate models. One secreted protein, Mtb8.4, induced a significant degree of protection (Table 2).

Nucleic acid immunization has proved an effective technique for delivering mycobacterial antigens as subunit vaccines. The initial experiments utilized DNA expressing *M. tuberculosis* Ag85A and *Mycobacterium leprae* heat shock protein (Hsp) 65 or the 36 kDa proline-rich antigen, and demonstrated protection against both aerosol and systemic expressing *M. tuberculosis* infection. Subsequently, plasmids expressing Ag85B, ESAT-6, and a number of secreted and non-secreted proteins have been shown to induce protective T cell responses (Table 3). Immunization with DNA-Ag85B induced strong antigen-specific Th1-like IFN-γ response to CD4 T cells and a high titre, specific IgG antibodies, but no measurable IL-4 response. The protective effect of DNA immunization correlated with the early recruitment of IFN-γ-secreting CD4 T cells to the lungs with 10 days of aerosol exposure. In contrast to BCG, DNA immunization also induced a CD8 T cells, which were both cytolytic and IFN-γ-secreting. This may contribute to their protective efficacy, as IFN-γ-secreting CD8 T cells are essential for protection against *M. tuberculosis* infection. Therefore any candidate antigen(s) should contain both CD4 and CD8 T cell epitopes, which can be presented by multiple MHC haplotypes. The effect of the DNA-delivered gene product may be increased by fusing it to a eukaryotic secretion signal sequence. Fusion of ESAT-6 and MPT64 with the tissue plasminogen activator signal sequence increased their protective effects, however, this was not observed with Ag85B (Kamath, unpubl. obs.).

Some general principles about subunit immunization against TB have emerged from both the protein and DNA vaccines. First, a single gene product can induce partial protection, but there is a hierarchy in the effect of different proteins (Fig. 1). This may indicate that the more effective antigens are encountered early during infection and therefore recall a response to these limits bacterial replication more effectively. Second, not all proteins induce protective immunity. For example, a-crystallin, whose expression is increased during dormancy, failed to protect despite being immunogenic (Triccas, unpubl. obs.). Moreover, the phosphate transporter, PstS1 which is a dominant antigen in the human response to *M. tuberculosis* infection, was found to be protective by one, but not another, group. Third, the protection conferred by any single antigen has been less than that of BCG in most cases, with the exceptions occurring when BCG induced relatively low levels of protection in the lungs (Table 3). In particular, BCG induced significantly greater protection against disseminated infection in the spleen than the subunit vaccines (Table 3).

Finally, immunization with more than one gene product is likely to be more effective vaccine in outbred populations (Table 4). A combination of DNA vaccines expressing three secreted proteins, which are commonly recognized during human infection, induced significantly greater protection than any one vaccine or combination of two. This effect was evident in the lungs after aerosol infection and at a site of dissemination in the spleen (Fig. 1). A separate approach used a combination of multiple DNA vaccines, each expressing two or more fused genes, for secreted and cytoplasmic proteins. Again the protective effect was increased, particularly in the spleen (Table 4). One advantage of subunit immunization is that it can be used to focus the immune response on one or a limited number of dominant, *M. tuberculosis*-specific proteins. This may avoid prior exposure to cross-reactive mycobacterial antigens shared with environmental mycobacteria blocking the induction of protective responses to *M. tuberculosis*-specific antigens. This will need to be balanced with providing sufficient T cell epitopes on different proteins to provide an adequate T cell repertoire.

The degree of protection conferred by DNA vaccines may be influenced by the relative virulence of the mycobacterial

---

Table 2 Comparison of the protective efficacies of protein vaccines with BCG against *Mycobacterium tuberculosis* infection

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Route of infection</th>
<th>Strain of mice</th>
<th>Time before challenge</th>
<th>Protection in lungs (log$_{10}$)</th>
<th>Protection in spleen (log$_{10}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFPP (IFA)</td>
<td>aerosol</td>
<td>B6D2</td>
<td>6–8</td>
<td>1.0*</td>
<td>1.2*</td>
</tr>
<tr>
<td>CFPP (DDA)</td>
<td>i.v.</td>
<td>C57Bl6</td>
<td>12–14</td>
<td>1.24</td>
<td>1.64</td>
</tr>
<tr>
<td>CFPP (MPL)</td>
<td>aerosol</td>
<td>C57Bl6</td>
<td>4</td>
<td>0.27†</td>
<td>1.34†</td>
</tr>
<tr>
<td>CFPP (MPL + IL12 + IL2)</td>
<td>aerosol</td>
<td>C57Bl6</td>
<td>4</td>
<td>0.9†</td>
<td>1.34†</td>
</tr>
<tr>
<td>ST-CPFP (MPL)</td>
<td>aerosol</td>
<td>C57Bl6</td>
<td>6</td>
<td>0.59†</td>
<td>0.58†</td>
</tr>
<tr>
<td>ESAT-6 (DDA + MPL)</td>
<td>aerosol</td>
<td>C57Bl6</td>
<td>6</td>
<td>0.68†</td>
<td>0.58†</td>
</tr>
<tr>
<td>85B (DDA + MPL)</td>
<td>aerosol</td>
<td>C57Bl6</td>
<td>6</td>
<td>0.3†</td>
<td>0.25†</td>
</tr>
<tr>
<td>85B-ESAT-6 (fusion) (DDA + MPL)</td>
<td>aerosol</td>
<td>C57Bl6</td>
<td>6</td>
<td>0.72†</td>
<td>0.7†</td>
</tr>
<tr>
<td>Mtb8.4 (IFA)</td>
<td>i.v.</td>
<td>C57Bl6</td>
<td>3</td>
<td>1.0</td>
<td>ND</td>
</tr>
<tr>
<td>85 A (MPL)</td>
<td>i.v.</td>
<td>C57Bl6</td>
<td>8</td>
<td>0.1</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* 3 weeks postinfection; † 6 weeks postinfection.

DDA, dimethyl dioctadecylammonium bromide; IFA, incomplete Freund’s adjuvant; MPL, monophosphoryl lipid; ND, not done.
Improving vaccines against tuberculosis

For example, plasmids expressing the immunodominant 35 kDa protein (MMPI), shared by *M. leprae* and *M. avium*, induced strong protective immunity against both *M. avium* and *M. leprae* in mice, which was equivalent to that of BCG in both cases. Bacille Calmette-Guérin protects against human leprosy infection, and any future subunit vaccines against TB should retain this important public health effect. In fact, DNA expressing Ag85B from *M. tuberculosis* does protect against both *M. leprae* and *M. ulcerans*, the cause of Buruli ulcer.

An important application of new anti-TB vaccines may be as postexposure vaccines. Reducing the proportion of the 2 billion subjects already infected with *M. tuberculosis* who will develop active TB would have a major impact on the TB epidemic. Mice previously infected with *M. tuberculosis* were immunized with a DNA vaccine expressing the *M. leprae* Hsp65, which is 95% homologous with *M. tuberculosis* GroEL, and this resulted in a significant reduction in the mycobacterial load. Subsequently, immunization with a plasmid expressing the *M. tuberculosis* Hsp65 was found to be less effective as a vaccine. Nevertheless, these results demonstrate the potential of subunit vaccines as potential immunotherapeutic agents.

### Table 3 Comparison of the protective efficacy of DNA vaccines expressing different Mycobacterium tuberculosis proteins with BCG against *M. tuberculosis* infection

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Route of infection</th>
<th>Strain of mice</th>
<th>Time before challenge</th>
<th>Protection in lungs (log&lt;sub&gt;10&lt;/sub&gt;) DNA BCG</th>
<th>Protection in spleen (log&lt;sub&gt;10&lt;/sub&gt;) DNA BCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secreted proteins</td>
<td>85 A aerosol</td>
<td>C57Bl6</td>
<td>3–10</td>
<td>1.2 ND</td>
<td>ND ND&lt;sup&gt;44&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>i.v.</td>
<td>C57Bl6</td>
<td>8</td>
<td>0.45 1.57</td>
<td>ND ND&lt;sup&gt;42&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>85B aerosol</td>
<td>C57Bl6</td>
<td>4</td>
<td>0.5 0.9</td>
<td>ND ND&lt;sup&gt;45&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>aerosol</td>
<td>C57Bl6</td>
<td>4</td>
<td>0.45 1.0</td>
<td>0.25 1.2&lt;sup&gt;46&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>i.p. BALB/c</td>
<td>3</td>
<td>&lt;0.5 &gt;1.0</td>
<td>1.4 1.5&lt;sup&gt;47&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ESAT-6 aerosol</td>
<td>C57Bl6</td>
<td>4</td>
<td>0.65* 1.0*</td>
<td>ND ND&lt;sup&gt;45&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>i.p. BALB/c</td>
<td>3–4</td>
<td>1.0 1.5</td>
<td>0.6 2.3&lt;sup&gt;48&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PstS1 i.p.</td>
<td>C57Bl6</td>
<td>2</td>
<td>0.6 1.0</td>
<td>0.4† 0.75&lt;sup&gt;49&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>i.v.</td>
<td>C57Bl6</td>
<td>8</td>
<td>0 1.4</td>
<td>0 2.0&lt;sup&gt;50&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>PstS2 i.v.</td>
<td>C57Bl6</td>
<td>8</td>
<td>0.5§ 1.4§</td>
<td>0.6 2.0&lt;sup&gt;50&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>PstS3 i.v.</td>
<td>C57Bl6</td>
<td>8</td>
<td>1.4** 1.4**</td>
<td>1.2** 1.2&lt;sup&gt;50&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MPT64 aerosol</td>
<td>C57Bl6</td>
<td>4</td>
<td>0.25 1.0</td>
<td>&lt;0.2 1.2&lt;sup&gt;45&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MPT64† aerosol</td>
<td>C57Bl6</td>
<td>4</td>
<td>0.8 1.5</td>
<td>0.4 2.3&lt;sup&gt;48&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MPT63 aerosol</td>
<td>C57Bl6</td>
<td>4</td>
<td>0.5 1.25</td>
<td>0.7 2.1&lt;sup&gt;46&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MPT32 aerosol</td>
<td>C57Bl6</td>
<td>4</td>
<td>0 1.25</td>
<td>0.2 1.2&lt;sup&gt;54&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Mtb39 aerosol</td>
<td>C57Bl6</td>
<td>4</td>
<td>0.5 0.75</td>
<td>ND ND&lt;sup&gt;51&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MTB41 aerosol</td>
<td>C57Bl6</td>
<td>4</td>
<td>0.9 1.1</td>
<td>ND ND&lt;sup&gt;43&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Mtb8.4 i.v.</td>
<td>C57Bl6</td>
<td>3</td>
<td>0.7 0.8</td>
<td>1.0 1.0&lt;sup&gt;41&lt;/sup&gt;</td>
</tr>
<tr>
<td>Heat shock proteins</td>
<td>Hsp65 i.p. Outbred parkes</td>
<td>3–4</td>
<td>0.5 1.0</td>
<td>0.4 1.5&lt;sup&gt;52&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>i.p. Outbred parkes</td>
<td>3–4</td>
<td>0.5 1.0</td>
<td>0.4 1.5&lt;sup&gt;52&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>i.p. CBA/B10</td>
<td>3–4</td>
<td>1.3 1.3</td>
<td>2.2 2.2&lt;sup&gt;52&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>i.p. BALB/c</td>
<td>3–4</td>
<td>1.6 1.8</td>
<td>1.4 1.8&lt;sup&gt;52&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hsp70 i.p. BALB/c</td>
<td>3</td>
<td>&lt;0.2 &gt;1.0</td>
<td>1.0 1.5&lt;sup&gt;47&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α-crystallin</td>
<td>aerosol</td>
<td>C57Bl6</td>
<td>4</td>
<td>0 1.25</td>
</tr>
<tr>
<td></td>
<td>Others i.p. Outbred parkes</td>
<td>3–4</td>
<td>0 1.0</td>
<td>0 1.5&lt;sup&gt;52&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>36 kDa i.p. Outbred parkes</td>
<td>3–4</td>
<td>0 1.0</td>
<td>0 1.5&lt;sup&gt;52&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>i.p. CBA/B10</td>
<td>3–4</td>
<td>1.1 1.3</td>
<td>1.8 1.8&lt;sup&gt;52&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>i.p. BALB/c</td>
<td>3–4</td>
<td>1.4 1.8</td>
<td>1.0 1.8&lt;sup&gt;52&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KatG† aerosol</td>
<td>C57Bl6</td>
<td>4</td>
<td>0.65 1.5</td>
<td>0.7 2.3&lt;sup&gt;48&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1818c PE aerosol</td>
<td>C57Bl6</td>
<td>5</td>
<td>0.5 1.0</td>
<td>0.8 1.4&lt;sup&gt;53&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Relative protective response normalized to Bacille Calmette-Guérin (BCG); † with tissue plasminogen activator- (TPA) signalling; ‡ 12 weeks postinfection, no protection at 4 weeks; § 10 weeks postinfection, no protection at 4 weeks; ¶ 10 weeks postinfection; ** 8 weeks postinfection; ND, not done.

Pathogen. For example, plasmids expressing the immunodominant 35 kDa protein (MMPI), shared by *M. leprae* and *M. avium*, induced strong protective immunity against both *M. avium* and *M. leprae* in mice, which was equivalent to that of BCG in both cases. Bacille Calmette-Guérin protects against human leprosy infection, and any future subunit vaccines against TB should retain this important public health effect. In fact, DNA expressing Ag85B from *M. tuberculosis* does protect against both *M. leprae* and *M. ulcerans*, the cause of Buruli ulcer.

An important application of new anti-TB vaccines may be as postexposure vaccines. Reducing the proportion of the 2 billion subjects already infected with *M. tuberculosis* who will develop active TB would have a major impact on the TB epidemic. Mice previously infected with *M. tuberculosis* were immunized with a DNA vaccine expressing the *M. leprae* Hsp65, which is 95% homologous with *M. tuberculosis* GroEL, and this resulted in a significant reduction in the mycobacterial load. Subsequently, immunization with a plasmid expressing the *M. tuberculosis* Hsp65 was found to be less effective as a vaccine. Nevertheless, these results demonstrate the potential of subunit vaccines as potential immunotherapeutic agents.

### Improving subunit vaccines

Although the results with subunit vaccines are encouraging, their protective effects should either exceed or be undoubtedly equivalent to that of BCG before consideration for human trials. Moreover DNA vaccines have been more effective in mice than humans, possibly because of differences in the optimal immuno-stimulatory oligonucleotides between the two species. Therefore a variety of approaches for increasing the efficacy of both DNA and protein vaccines have been explored.
First, the adjuvant effects of cytokines on both DNA and protein vaccines have been tested (Table 4). The hetero-
dimeric cytokine, IL-12, is produced by DC and macrophages
and is essential for the development of IFN-γ-secreting Th1-
like CD4 T cells. Mice deficient in the p40 chain of IL-12,
which is shared by IL-12 and the recently described cytokine
IL-23, are profoundly susceptible to *M. tuberculosis*-infection,
while mice deficient in the IL-12 p35 chain alone are partially
compromised. Therefore we developed a plasmid vector
which encoded both chains of murine IL-12 between the self-
cleaving peptide fragment of the foot and mouth disease virus
2 A protein. Transfection of cell lines with this plasmid
resulted in the coordinate expression of both chains of IL-12
and the secretion of functional cytokine. Co-immunization of
mice with this IL-12 plasmid and DNA-85B resulted in
increased frequency of Ag85B-specific CD4 T cells and a
reduction in the anti-Ag85B IgG titre, compared to recipients
of DNA-85B. Following *M. tuberculosis* challenge, there
was an increased protective effect in the lung, to the level
observed with BCG (Fig. 2), and in the spleen (Table 4). Co-
immunization with the IL-12 plasmid and DNA-35 also
resulted in increased protection against *M. avium* infection to
a level exceeding that achieved by BCG immunization (Mar-
tin, unpubl. obs.). Interleukin-12 as a protein adjuvant also
increased the protective effect conferred by a mixture of
*M. tuberculosis* culture filtrate proteins (Table 2).

Interleukin-18 bears some structural similarities to IL-1β
and is also secreted as a pro-inflammatory cytokine by DC
and macrophages. It also potentiates the development of Th1-
like T cells and synergizes with IL-12 to stimulate maximum
IFN-γ production from T cells. Therefore, we analysed the
relative effects of coimmunization of IL-18 and IL-12-
expressing plasmids with DNA-85B on IFN-γ responses and
protective immunity. Codelivery of IL-18-producing vac-
cines increased the specific IFN-γ T-cell responses, but did
not increase the protective effect of DNA-85B, or further
enhance the adjuvant effects of IL-12 (Table 4).

Not all manipulations which increase the IFN-γ responses
to vaccine antigens necessarily increase the protective effi-
cacy of the anti-TB vaccine. For example, coimmunization of
mice with a plasmid expressing GM-CSF and DNA-85B
enhanced IFN-γ T cell responses, with no change in the
protective effect. Interestingly there was an increase in the
anti-Ag85B IgG response with GM-CSF as the adjuvant,

Table 4 Strategies to enhance the protective efficacy of DNA vaccines against *Mycobacterium tuberculosis* infection

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Route of infection</th>
<th>Strain of mice</th>
<th>Time before challenge</th>
<th>Protection in lungs (log_{10})</th>
<th>Protection in spleen (log_{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DNA</td>
<td>BCG</td>
</tr>
<tr>
<td><strong>Cytokine adjuvants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>85B + pIL-12</td>
<td>aerosol</td>
<td>C57Bl6</td>
<td>6</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>85B + pIL-18</td>
<td>aerosol</td>
<td>C57Bl6</td>
<td>4</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>85B + pIL12 + pIL18</td>
<td>aerosol</td>
<td>C57Bl6</td>
<td>4</td>
<td>0.7</td>
<td>1.2</td>
</tr>
<tr>
<td>85B + pGMCSF</td>
<td>aerosol</td>
<td>C57Bl6</td>
<td>4</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Combination of multiple antigens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mpt64, ESAT-6, 85B</td>
<td>aerosol</td>
<td>C57Bl6</td>
<td>4</td>
<td>0.6</td>
<td>1.1</td>
</tr>
<tr>
<td>TPA combo*</td>
<td>aerosol</td>
<td>C57Bl6</td>
<td>5</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.73†</td>
<td>0.63†</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td>i.v.</td>
<td>C57Bl6</td>
<td>6</td>
<td>0.85</td>
<td>1.7</td>
</tr>
</tbody>
</table>

<sup>*ESAT-6, mpt63, mpt8e, 85B, katG, mtb12, mth 8.4, *Mycobacterium tuberculosis* (MTB 39); 1818c<sup>e</sup> fused to tissue plasminogen activator- (TPA) signalling. †16 weeks post challenge; pGMCSF, plasmid granulocyte macrophage colony stimulating factor. \</sup>
compared to the fall observed with IL-12, suggesting that when both cellular and humoral immunity are stimulated, protection against mycobacterial challenge is not necessarily increased. A similar pattern has also been observed with other non-cytokine-based approaches to increasing the responses to DNA vaccines (Palendira, unpubl. obs.). This highlights the fact that changes in the IFN-γ T cell responses alone do not necessarily correlate with protection following *M. tuberculosis* infection.

An alternative approach is to combine DNA immunization with another vector or protein antigen in a prime/boost schedule (Table 5). Combinations of DNA and attenuated pox-virus vectors increased the protection of both Simian immuno-deficiency virus (SIV) and malaria vaccines.\(^{48,69}\) Initially we primed with DNA expressing Ag85B or MPT64 and boosted with vaccinia or modified vaccinia ankara (MVA) vaccines expressing the same respective antigens.\(^{66,70}\) Although this increased the epitope-specific CD8\(^+\) T cell response with either combination, it did not increase protection against *M. tuberculosis* challenge (Table 4). By comparison, when we primed with DNA-85B and boosted with BCG, there was a marked increase in the protective effect in both the lungs and especially the spleen, with the bacterial loads significantly less than in BCG-immunized mice boosted with BCG (Fig. 3).\(^{66}\) This effect was robust and was not further enhanced by the addition of plasmid IL-12 to the DNA priming dose.\(^{56}\) The enhanced protection following the DNA/BCG prime/boost immunization was in part dependent on the increased CD8 T cell responses, as treatment with anti-CD8 mAbs during *M. tuberculosis* challenge partially abrogated the effect.\(^{66}\) By contrast, DNA priming and boosting with Ag85B as protein did not increase protection in these experiments, although others have reported a slight increase in the protective effect of DNA immunization with protein boosting.\(^{42}\) In separate experiments priming with DNA-85B and boosting with MVA-Ag85B was reported to increase protection.\(^{67}\)

An important conclusion from these experiments is that the level of protection in the mouse model of experimental TB is not limited to that achieved with BCG alone. Improved protection requires increased expansion in both the CD4\(^+\) and CD8\(^+\) T cell compartments. A particular contribution of the DNA vector is to focus the immune response on an immunodominant antigen in BCG and to stimulate antigen-specific CD8\(^+\) T cells, which usually respond only weakly to BCG immunization alone. A challenge now is to determine if this increased degree of protection can be achieved with enhanced individual vaccine vectors or combinations that do not utilize BCG or other viable vaccine vectors.

**Application of new vaccines in humans**

The extensive laboratory studies on candidate vaccines described above have been accompanied by renewed interest in vaccine trials in humans. The largest recent study was the randomized controlled Karonga trial of the addition of dead...
effectiveness of BCG against pulmonary as well as disseminated adult leprosy with an additional 50% protection from a second passage of this attenuated strain of BCG in a hyperendemic area in South Africa. Therefore another study is examining the effect of different strains of BCG in a hyperendemic area in South Africa. Initial studies with M. vaccae, a saprophytic mycobacterium, suggested it had immunotherapeutic benefits. However a recent controlled study showed that M. vaccae did not provide additional benefit to the clearance of mycobacteria from patients with pulmonary TB receiving antibiotic therapy.

What are the requirements for testing the new candidate vaccines in humans if preclinical studies confirm that they have equivalent or increased protective efficacy to BCG? The most important need is a better immunological correlate of protective immunity against mycobacterial infections. Tuberculin skin testing for delayed type hypersensitivity did not correlate with protective immunity in the MRC trial. Antigen-specific IFN-γ T cell responses are useful for confirming the induction of the pattern of cellular immunity associated with protection, and are widely used in animal studies. These will be employed in the initial Phase I/II human studies of vaccines, but it should be emphasized that increased IFN-γ production and protection do not always correlate in experimental models of TB. Interferon-γ alone is not sufficient to activate human infected macrophages to kill M. tuberculosis, and a more global in vitro mycobacterial killing assay may measure the effect of the multiple factors needed to control the infection. A potential assay used lucitherase expression in transfected BCG as a rapid measure of viability. Circulating lymphocytes from tuberculin skin test positive immune individuals activated infected macrophages to inhibit light production from lucitherase-expressing BCG, and this may correlate with the in vitro killing of the mycobacteria.

There are important practical and ethical considerations in the design and implementation of TB vaccine trials. The sites must have a high incidence of TB to measure the protective effect and the population must be stable to ensure continued follow-up over the long periods necessary for the clinical trials. A functioning TB control program is essential to provide antibiotic treatment for those who develop disease over the course of the trial. An important confounding factor will be the effects of HIV infection and consequent immunodeficiency on the measurement of the vaccine efficacy. Therefore communities with a relatively low HIV prevalence rate may be more suitable. This reinforces the importance of the interaction of HIV and M. tuberculosis in individuals and communities, and the potential effect this will have on the interpretation of drug treatment or vaccine trials for each infection. The design of interventions to control each disease will be affected by changes in the prevalence or treatment programs for the other condition. The requirements for monitoring both conditions are not dissimilar and future studies will be dependent on staff and field centres with expertise in both disease. The challenge to the research and medical communities is to obtain the resources and the political will necessary to enable the testing of the emerging vaccine candidates for both these human pathogens.

**Acknowledgements**

Financial support is gratefully acknowledged from the National Health and Medical Research Council of Australia, the Community Health and Anti-Tuberculosis Association, the Tropical Disease Research Program of the World Health Organization and the NSW Department of Health through its infrastructure grant. UP is supported by an Australian Postgraduate Award and the Cooperative Research Centre for Vaccine Technology. We thank Rachel Pinto for the preparation of Table 1, Dr Arun Kamath for the provision of unpublished data in Fig. 1, and Drs James Triccas and Caroline Demangel for helpful discussions.

**References**

Improving vaccines against tuberculosis


65 Cooper AM, Kipnis A, Turner J, Magram J, Ferrante J, Orme IM. Mice lacking bioactive IL-12 can generate protective, antigen-specific cellular responses to mycobacterial infection only if the IL-12 p40 subunit is present. J. Immunol. 2002; 168: 1322–7.


