Despite sometimes not being recognized, the direct and indirect costs of TB, and the social consequences, are often catastrophic for the individual patient, the family, and the wider community

All Is Running – Angel Ramírez
Sculpture
(224 x 200 cm)

'But it wasn't! This day never existed
in any calendar of the world!...
Really, never existed. Life is good.'

The Fear (Poem) – Dulce María Loynaz

Angel Ramírez
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Angel Ramírez graduated from National School of Arts and from the Higher Institute of Art, both in Havana.

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DNA Vaccines for the Prophylaxis of Tuberculosis

Marta Romano and Kris Huygen

Abstract

Plasmid DNA vaccination is a powerful and easy method for the induction of strong humoral and CMI responses in mice. By virtue of its strong capacity to induce CD4+ mediated Th1 and CD8+ mediated CTL responses, this vaccine approach is particularly attractive for the prophylaxis of intracellular pathogens, such as MTB and other pathogenic mycobacteria. DNA vaccination has also been successfully applied for the characterization of immunodominant, human T cell epitopes using HLA-transgenic mice. In small rodents, the potential of mycobacterial DNA vaccines is well established but in humans and larger animals, DNA vaccines are less immunogenic. In vivo electroporation and the use of appropriate costimulants that trigger dendritic cells may help to overcome this low immunogenicity. Also, DNA vaccines appear to be particularly effective for priming immune memory and subsequent boosting with recombinant protein, recombinant pox-viruses or with *M. bovis* BCG vaccine are particularly promising for future applications. Although DNA vaccines targeting TB or other mycobacterial diseases have not been tested in humans so far, major breakthroughs in the field of cancer and AIDS and the licensing of three DNA vaccines for veterinary use give reasonable hope that these vaccines will get into the clinical pipeline sooner or later. The most likely formulation that may eventually reach Phase I and Phase II clinical testing, may be a combination of plasmid DNA—encoding protective (fusion)-proteins or a string-of beads poly-epitope—with the existing *M. bovis* BCG vaccine.

Vaccines protecting at the same time against primary TB and against reactivation of latent TB will undoubtedly have the biggest impact on the control of this infectious disease.

Introduction

Almost 20 years ago, Felgner and colleagues described for the first time that intramuscular injection of naked plasmid DNA encoding the bacterial enzyme β-galactosidase could lead to direct gene transfer to myocytes, transcription in the nucleus, and subsequent synthesis of the enzyme by the muscle cell (1). In 1992, Tang demonstrated that this plasmid injection could elicit an immune response (2) and the next year it was shown that mice (3) and chickens (4) injected with influenza DNA could be protected against subsequent viral challenge. Since then, DNA vaccines have been reported to induce protective immunity in numerous animal models of parasitic, viral, and bacterial diseases (5–7). This type of vaccine also holds promise for the treatment of cancer (8), allergies (9), and autoimmune diseases (10). Conceptually, DNA vaccines have a number of potential advantages when compared to current vaccines inducing CMI (which are mostly live attenuated pathogens): ease of preparation, stability, and safety for the immunocompromised host. Polynucleotide vaccines do not induce vector immunity and can therefore be used for repeated boosting. Also they do not require a cold chain for storage as they can be stored in a dry, precipitated form. This is particularly important for vaccines designed for developing countries, where maintenance of a cold chain can often not be guaranteed.

Immune Responses Induced with DNA Vaccines

In a DNA vaccine, the gene encoding an antigen is inserted into a bacterial plasmid vector, which is amplified in transformed bacteria, and the purified plasmid DNA is administered to an immunocompetent host. Priming of the immune response involves professional antigen presenting cells (APCs), such as dendritic cells and Langerhans cells, that endocytose DNA into acidic vesicles for subsequent transport to the nucleus, followed by transcription into mRNA and protein translation in the cytoplasm. Bacterial plasmid DNA contains unmethylated CpG sequences that act as polyclonal activator of B cells in vitro and as adjuvant in vivo. These CpG motifs stimulate production of co-stimulatory molecules by APCs through interaction with a specific intracellular TLR, that is, TLR-9,

present on the surface of the early endosome (11). On the other hand, besides immunostimulatory CpG motifs there also exist immunoinhibitory ones and the latter motifs have been used for induction of tolerance in DNA vaccines targeting autoimmune diseases (10). Monocyte/macrophages, NK cells, and B lymphocytes also express the TLR-9 receptor and it has been postulated by Lanzavecchia that CpG motifs from ubiquitous bacteria could continuously stimulate memory B cells and hence play a role in the maintenance of serological memory (12). The major co-stimulatory cytokines induced upon TLR-9 triggering are IL-12 (which stimulates NK cells to produce IFN-γ and favours the development of a Th1 type T helper subset), TNF-α, and IL-6 (which favours antibody production but also plays a role in cytotoxic T lymphocyte (CTL) and Th_{IL-17} differentiation). Following DNA vaccination, antigenic material is generated in the myocyte/keratinocyte but also within the APC (5), and exogenous and endogenous antigen processing can proceed in much the same way as following infection with intracellular pathogens. DNA vaccines stimulate both the exogenous (MHC class II restricted) and the endogenous (MHC class I restricted) antigen presentation pathway (5). Dendritic cells can also take up antigen-containing apoptotic bodies from transfected myocytes and present the relevant peptides to CD4+ and CD8+ T cells (the so-called cross-priming phenomenon) (13). By virtue of this induction of CD8+ T cell responses, DNA vaccines mimic strongly the infection with live pathogens, in contrast to vaccines based on protein antigens or killed pathogens that are preferentially processed through the exogenous presentation pathway generating only MHC class II restricted CD4+ responses. It is particularly this class I restricted presentation, resulting in strong CD8+ mediated immune responses, that is a hallmark of DNA vaccines and that makes them particularly attractive as vaccine formulations against viruses and intracellular bacteria, such as mycobacteria.

DNA Vaccines against Tuberculosis

Between March 2006 and November 2008, the number of hits in a PubMed search using the key words 'DNA vaccines+ tuberculosis' doubled from 250 to 497! Within the scope of this chapter, it is impossible to discuss all these papers, and I refer to a couple of my previous reviews for the less recent literature (14–16). Here we will highlight mostly the new findings published between 2006 and 2008.

In 1996, the group of Jo Colston and Douglas Lowrie at the Medical Research Council, London, and our group at the former Pasteur Institute in Brussels (now integrated in the Scientific Institute of Public Health), were the first to report on the use of DNA vaccines against TB, using DNA encoding hsp65 of

M. leprae and Ag85A of MTB respectively (17, 18). Twelve years later, it is well established that intramuscular immunization of mice with plasmid DNA encoding mycobacterial antigens is a potent inducer of strong T helper type immune responses, characterized by high levels of IL-2 and IFN-γ and little or no IL-4/IL-5 in antigen stimulated spleen cell cultures. A wide variety of potential TB vaccine candidates have been defined using this technique. Because pathogenic mycobacteria remain largely confined to the phagosome in experimentally infected mice, it has been very difficult to identify cognate specificities of CD8+ mediated immune responses in these models. As such, DNA vaccination has been a valuable tool for the characterization of mouse MHC class I restricted epitopes for mycobacterial antigens, such as the mycolyl-transferases of the Ag85 complex (19), the phosphate-binding proteins PstS-1 (20) and PstS-3 (21), MTB32 component of the 72F fusion protein (22), and the latency-associated Rv2626c protein encoded by the dormancy DosR regulon (23). Immunization of mice with plasmid DNA encoding the MTB41 gene sequence resulted in the development of antigen-specific CD4+ and CD8+ T cells, and protection against challenge with virulent MTB. However, in contrast to DNA immunization, strong MTB41-specific CD4+ T cell response, but no MHC class I restricted CTL activity, was detected in the spleen cells of infected mice; therefore, suggesting that the induction of CD8+ T cell response to MTB41 epitopes by DNA immunization may not be relevant to protection because these epitopes are not recognized during the infectious process (24). However, in most experimental mouse studies, demonstration of cognate CD8+ mediated responses has been very difficult, possibly because of the delayed appearance of these cells. (25) At least for PstS-3, we were able to demonstrate spleen cell IFN-γ responses and in vivo CTL activity directed against the MHC class I (D^b) restricted epitope identified by DNA vaccination in TB infected mice (21).

Among the best documented mycobacterial plasmids are the DNA vaccines encoding the mycolyl-transferases Ag85A and Ag85B. Plasmids encoding these highly conserved, mycobacteria-specific antigens, have a definite vaccine potential not only for human TB (18), but also for Buruli ulcer caused by *Mycobacterium ulcerans* (26, 27), for leprosy caused by *M. leprae* (28), for *M. avium* subsp. *avium* (29), and *M. avium* subsp. *paratuberculosis* infections (30). Very strong Th1 type immune responses can be induced in mice with these plasmids (31) and Ag85 specific CD4+ T helper cells induced by the vaccine can even function as novel adjuvants for the effective induction of HIV-1 specific CTL responses (32).

Members of the PE-PPE family have also been reported to have a strong vaccine potential. The PPE protein family of MTB includes 69 proteins rich in glycine and, together with the PE (Pro-Glu) protein family, accounts for approximately 10 per cent of the coding capacity of the MTB genome. There is little

functional information about PPE proteins, but their polymorphic nature suggests that they may represent antigens of immunological relevance (33). Subcellular fractionation and immunoelectron microscopy studies have indicated that some PPE proteins are located at the periphery of the bacterial cell and could therefore be accessible to the host immune system. Moreover, they induce strong immune responses in animals and humans infected with MTB (34). We have evaluated the vaccine potential of PPE44 (Rv2770c), overexpressed in virulent MTB H37Rv as compared with the attenuated H37Ra strain (35). *Ppe44* gene expression shows high quantitative variations in clinical isolates selected to represent the major phylogenetic lineages of the MTB complex and, more specifically, strains of Beijing type demonstrate high *ppe44* expression (36). PPE44-specific immune responses could be detected in mice acutely, chronically, and latently infected with MTB. Vaccination of mice with a plasmid DNA vaccine coding for PPE44 and of recombinant PPE44 protein formulated in adjuvant generated strong cellular and humoral immune responses, and immunodominant T cell epitopes were identified. Most importantly, vaccination of mice with both subunit vaccines followed by an intratracheal challenge with MTB resulted in a protective efficacy comparable to the one afforded by BCG (37).

Chaitra et al. reported on DNA vaccines encoding two PE-PGRS proteins, encoded by Rv3812c and Rv3018c respectively. Strong immunogenicity was demonstrated in BALB/c mice, both against MHC class II and MHC class I restricted epitopes. Interestingly, an epitope-specific response was demonstrated by the lysis of peptide-pulsed APCs, release of perforin, and IFN-γ production (38). Finally, Singh et al. reported recently on DNA vaccination encoding three MTB proteins, namely PE_PGRS 16 (Rv0977), PE_PGRS 26 (Rv1441c), and PE_PGRS 33 (Rv1818c). All three PE_PGRS proteins were found to be cell-surface antigens, but immunization of mice with these PE_PGRS antigens as DNA vaccines showed no protection in a TB aerosol challenge model (39).

Optimalization of Tuberculosis DNA Vaccines

Attempts to Increase Delivery to Antigen Presenting Cells

A major problem with DNA vaccines is their transfection efficacy and the amount of actual protein synthesized. In vivo electroporation increases the number of DNA transfected myocytes dramatically and, both in mice and farmed ruminants, this electroporation technique was shown to increase the immunogenicity of TB DNA vaccines (40, 41). Li et al. showed that an in vivo DNA electroporation prime and protein boost strategy can also enhance humoral immunity of TB DNA vaccines encoding Ag85A and ESAT-6 in non-human primates (42).

Cross-priming, in which antigen-containing apoptotic bodies from dead, plasmid transfected muscle cells are engulfed by immature dendritic cells, is thought to be essential in the priming of the immune response upon intramuscular DNA vaccination (13). In an attempt to increase cross-presentation through apoptosis, we inserted the DNA encoding caspase-2 prodomain followed by wild-type or catalytically inactive mutated caspase-3 into a plasmid encoding Ag85A. Transient transfection showed that the mutated caspase induced slow apoptosis, normal protein expression, and NF-κB activation while wild-type caspase induced rapid apoptosis, lower protein expression, and no NF-κB activation. Ag85A specific antibody production was increased by co-expressing the mutated and decreased by co-expressing the wild-type caspase. Vaccination with pro-apoptotic plasmids triggered more Ag85A specific IFN-γ producing spleen cells, and more efficient IL-2 and IFN-γ producing memory cells in spleen and lungs after MTB challenge. Compared to DNA encoding secreted Ag85A, vaccination with DNA co-expressing wild-type caspase increased protection after infection with MTB, while vaccination with plasmid co-expressing mutated caspase was not protective, possibly due to the stimulation of IL-6, IL-10, and IL-17A production (43).

Complexation of DNA to adjuvants such as the cationic lipid vaxfectin™ (44) can increase immunogenicity and protective efficacy of DNA, but as this adjuvant is particularly effective for increasing antibody responses, through the stimulation of the Th2 type cytokine IL-6, its use needs to be studied carefully, particularly with respect to the possible induction of deleterious Th_{IL-17} cells (45). For mucosal delivery of DNA vaccines it is essential to protect them against host endonucleases by formulation in a carrier system. DNA vaccination has been successfully used for the definition of human MHC class I restricted epitopes, using HLA-A*0201 transgenic mice (46) and using these HLA-A*0201 transgenic mice, Bivas-Benita et al. demonstrated that pulmonary delivery of chitosan-DNA nanoparticles could induce (be it weak) spleen cell IFN-γ responses against four out of seven of the HLA-A2 predicted peptides encoded by a poly-epitope DNA (47). Manganelli and his colleagues have developed a non-pathogenic invasive commensal *Escherichia coli* BM2710 strain that can be used for the intranasal delivery of plasmid DNA encoding Ag85A and HtpX (48). Antigen-specific T cell responses and protection against MTB challenge were induced with DNA doses at least a hundredfold lower that those needed for intramuscular immunization. This mucosal approach using non-pathogenic *E. coli* for needle-free plasmid delivery certainly merits further studies.

Attempts to Increase Vector Immunogenicity

A promising approach to maximize expression of microbial genes in the plasmid is the optimization of codon usage. A synthetic humanized Ag85B

gene, codon-optimized for expression in human cells, was reported to display about sixfold increased in vitro expression in Cos-7 cells. Antibody responses were not affected by the optimization, but Th1-like and CTL responses (assessed in BALB/c mice) induced with the humanized gene were higher than with the plasmid encoding the wild-type Ag85B. Finally, reduced colony forming unit (CFU) counts in spleen and lungs from animals challenged with MTB four weeks after the third DNA immunization were also indicative of increased potency of the humanized pAg85B DNA (49).

Multi-subunit vaccination by co-immunization with different DNA vectors that are not very effective as single vaccines may result in a greater degree of protection as indicated by reduced CFU counts (50) and, more convincingly, even by up to sevenfold prolonged survival times following high dose aerosol challenge as compared to mice vaccinated with vector DNA only (51). For industrial purposes however, the use of hybrid genes or of multipromoter plasmids is more interesting than a combination of plasmids. In this line of thinking, Steven Reed and his colleagues at Corixa Corporation have shown very convincing results for a MTB72F DNA encoding a fusion of the MTB39 PPE protein and a 32kD serine protease (22). Similarly, Marta Romano in my group has analysed the use of a pBudCE4.1 vector encoding the genes for the mycolyl-transferase Ag85A and the 40 kDa phosphate-binding protein PstS-3, under control of the promoters for IE1 of CMV and of human elongation factor EF1α respectively. Although both antigens were expressed by the pBudCE4.1 vector, humoral and cellular immune responses clearly indicated an antigenic competition at the level of CD4+ (but not of CD8+) T cells, between the Ag85A and PstS-3 protein, with the mycolyl-transferase being the dominant partner over the phosphate transport receptor (52, 3). We hypothesize that prior activation of Ag85A-specific CD4$^+$ T cells directed against this common mycobacterial antigen leads to cross-competition for MHC class II-restricted peptide complexes of the Pst-3 antigen. This might have implications for future combination vaccines using components of the Ag85 complex. The group of Fadda et al. reported on a multigene DNA combination, encoding the combination of Ag85Bcoupled to ESAT-6 (54), a fusion protein that has entered into clinical Phase I and Phase II trials as Hybrid1 protein-subunit vaccine (55). They showed that this Ag85B–ESAT-6 vaccine could not be enhanced by broadening the antigen repertoire by adding other highly immunogenic secreted proteins (54), which is largely in agreement with our findings on the combination of Ag85A and PstS-3.

Another way to increase immunogenicity of DNA vaccines is the co-administration of plasmids encoding co-stimulatory molecules (CD80/CD86) or adjuvant cytokines. Co-immunization with plasmids expressing granulocyte-macrophage colony stimulating factor (GM-CSF) or IL-12 can enhance the

T cell immunity of DNA vaccines, encoding Ag85B or MPT64, about twofold. However, this is not sufficient to improve their protective efficacy at the peak of infection after an aerosol challenge with MTB (56, 57). DNA encoding IL-23, but not IL-27, was also reported to increase efficacy of DNA encoding Ag85B from MTB (58). Dou et al. addressed the immune adjuvant effects of IL-21 on a DNA vaccine encoding Ag85A. Their results showed that the DNA vaccine construct pRSC-IL21-Ag85A elicited stronger immune responses in BALB/c mice than pRSC-Ag85A (59). Finally, DNA encoding the cytokine IL-15, with a pivotal role in maintenance of memory T cells, holds particular promise (60), although to my knowledge, pIL-15 has not been tested in combination with mycobacterial antigens.

Prime-boost Strategies

By virtue of the strong Th1 biased and MHC class I restricted immune responses that DNA vaccines can induce, they are particularly attractive as priming agents in prime-boost regimens. For HIV and malaria these prime-boost regimens have progressed successfully to clinical trials (61).

Systemic boosting of DNA primed animals with mycobacterial proteins (62–65) or with recombinant Modified Vaccinia Ankara type (MVA) (66) or recombinant replication-deficient adenoviruses (67) have been reported to increase immunogenicity and/or protective efficacy of TB DNA vaccines. Similarly, boosting DNA primed mice with a live *Salmonella typhimurium* vaccine carrier, expressing ESAT-6, has also been attempted (68).

Zhou Xing and his colleagues have demonstrated recently that airway delivery of soluble Ag85A protein can restore protective mucosal immunity induced by single intramuscular Ag85A DNA vaccination. Recruitment of systemically activated antigen-specific T cells into the airway lumen of DNA vaccinated mice appears to be critically dependent not only on proinflammatory signals induced by the intranasal instillation but equally on parenteral immunization and robust systemic antigen-specific T cell priming, which is an important conceptual finding (69).

Combinations of Plasmid DNA with the Existing *M. Bovis* BCG Vaccine

Feng et al. were the first to report that sequential immunization with mycobacterial antigen 85B-expressing DNA and *M. bovis* BCG Tokyo was more effective than BCG immunization alone in protecting B6 mice against an aerosol MTB infection (70).

It is now well established that immune responses induced by the existing BCG vaccine can be augmented by combinations with plasmid DNA in mice, guinea pigs, and cattle either by priming with DNA (70–72) or by boosting

with DNA (73–75). In most of these studies, increased efficacy of BCG has been measured by CFU counting or pathological scoring in infected organs, but effects on long-term survival were rarely reported. Performing long-term survival studies in BALB/c mice infected intravenously with MTB, we have shown that priming with DNA prior to BCG, but not boosting after BCG with DNA (or recombinant protein or recombinant MVA for that matter) encoding Ag85A, could increase the potency of the BCG vaccine, resulting in seven to nine weeks longer mean survival times (76).

A BCG prime-DNA boost protocol, using plasmid encoding a 72F fusion protein, did increase the long-term survival of guinea pigs infected by a low dose aerosol challenge (75). The reason for the discrepancy with our report is not clear, but may be linked to the different animal species used.

It is not very likely that boosting with BCG after priming with DNA will be applicable as a vaccine regimen for developing countries, where neonatal BCG vaccination is part of the expanded programme of immunization by WHO. On the other hand, this regimen may have a better chance for testing in populations at risk (health care workers, prisoners, exposed household contacts) in developed Western countries where BCG is not routinely administered.

Post-Exposure and Therapeutic DNA Vaccines

Post-exposure TB vaccines are being developed to prevent disease in individuals latently infected with MTB. However, concerns about the potential induction of deleterious Koch-like reactions have limited progress in assessing the effectiveness of post-exposure vaccination. Derrick et al. reported in a comprehensive study on the safety of post-exposure vaccination with 12 different MTB preparations, among which were 4 DNA vaccines in C57BL/6 mice. In a low bacterial burden model (drug treatment for three months started one month after infection), post-exposure vaccination did not induce significant reactivation and only injection of BCG evoked increase in lung inflammatory responses at one month after the immunizations. Additionally, although significant increases in lung inflammation were seen for animals injected with the hsp65 DNA vaccine or a MTB culture filtrate, no differences in the survival periods were detected between vaccinated and non-vaccinated mice at 10 months post-vaccination (77). In an active disease model of immunotherapeutic vaccination, significantly more lung inflammation was observed one month after administration of hsp65 DNA but none of the vaccine formulations tested at this time increased (nor decreased) the lung bacterial burden at this early time point. Furthermore, vaccination of diseased mice with BCG or TB DNA vaccines did not significantly affect mortality rates compared to non-vaccinated controls (77). These results

are contradictory to different studies reported by Doug Lowrie and Celio Silva, who showed that MTB infected BALB/c mice given four doses of plasmid DNA encoding hsp65 of *M. leprae* demonstrated a rapid and spectacular decline in live bacteria in spleen and lungs up to five months later (78). On the other hand, they confirm indirectly the findings that Ag85A DNA vaccine, known to induce protective immunity and prevent long-term necrosis in guinea pigs, failed to protect mice when given in an immunotherapeutic model in mice earlier infected by aerosol with MTB (79). It is possible that the genetic background of the mice used in these studies (C57BL/6 versus BALB/c) and the origin of the hsp65 gene (MTB versus *M. leprae)* are responsible for these discrepancies. The use of DNA vaccines in the immunotherapy of TB therefore remains a controversial matter but we would refer the reader to a comprehensive review written by Lowrie in 2007 (80).

Finally, combinations of DNA vaccines with chemotherapy have been described. In a report by Ha et al., combinations of chemotherapy with a double-gene DNA vaccine, composed of Ag85A and PstS-3, completely blocked TB reactivation and significantly prevented a secondary infection when chemotherapy was combined simultaneously (81). Likewise, a report by Nuermberger et al. has suggested a beneficial effect of the combination of rifapentine, moxifloxacin, and DNA encoding hsp65 in a mouse model of latent MTB infection (82).

Clearly more work is needed in the field of post-exposure prophylaxis, which is hampered by the long duration time of the latency experiments. The actual BCG vaccine is not very effective in inducing immune responses against latency associated antigens such as HspX (Rv2031c) (83) and this could partially explain the low efficacy of BCG against pulmonary, reactivation TB (84). Combination of BCG with DNA vaccines encoding latency associated antigens (eventually coupled to DNA vaccines encoding early secreted antigens, effective against primary infection) is therefore a very tempting approach, but a proof of concept remains to be given.

Tuberculosis DNA Vaccines in Animals Other than Mice

DNA vaccines are very potent in a wide variety of experimental mouse models, but some promising results have also been obtained with mycobacterial DNA vaccines in guinea pigs and in 'bigger' animals such as cattle and sheep. Specifically for human TB, published literature has reported on DNA vaccines in guinea pigs and macaques.

Guinea Pigs

A number of reports have analysed the potency of TB DNA vaccines in guinea pigs. This animal species is often considered to be a better model for studying TB vaccines than the mouse because TB-infected guinea pigs demonstrate a necrotizing lung pathology similar to the one observed in humans, whereas in mice, MTB remains confined to closed granulomas, without caseation. Baldwin et al. have described that vaccination with DNA encoding Ag85A can prevent the onset of caseating disease, which is the hallmark of the aerogenic infection model in this species. Survival was prolonged in Ag85A DNA vaccinated guinea pigs but shorter than in BCG vaccinated animals (85). Vaccination of guinea pigs with DNA encoding the secreted antigen MPB83 reduced the severity of pulmonary lesions after a low dose *M. bovis* aerosol challenge, but did not protect the animals from haematogenous spread of bacilli to the spleen (86). Vaccination with Ag85A DNA exerted no measurable protective effect against infection with *M. bovis* in this study (86). The most promising results so far in guinea pigs have been obtained with DNA encoding the MTB72F fusion protein. Guinea pigs vaccinated with this fusion protein either as DNA or as recombinant protein and challenged by aerosol with virulent MTB showed prolonged survival times, comparable to those of BCG vaccinated animals (22). More importantly, co-administration of this MTB72F either as protein or as plasmid DNA together with the *M. bovis* BCG vaccine significantly prolonged survival time of guinea pigs challenged with MTB by low dose aerosol as compared to animals vaccinated with BCG alone (75).

Julia Vipond et al. selected a number of genes induced in response to in vivo-like stress stimuli, such as low oxygen and carbon starvation or growth in macrophages, and tested these candidates as plasmid DNA vaccines for their ability to protect against MTB challenge in a guinea pig aerosol infection model. Four vaccines encoding for proteins such as PE and PPE proteins, a zinc metalloprotease, and an acyltransferase, gave a level of protection that was statistically better than saline in the lungs (87).

Khera et al. analysed DNA vaccines expressing mycobacterial antigens ESAT-6 (Rv3875), alpha-crystallin (Rv2031c), and superoxide dismutase A (Rv3846) for protective efficacy in guinea pigs and showed that the DNA vaccine expressing superoxide dismutase imparted the maximum protection as observed by a 50- and 10-fold reduction in bacillary load in spleens and lungs, respectively, in comparison to immunization with vector control (88).

De Paula et al. co-encapsulated DNA hsp65 and the adjuvant TDM into biodegradable poly(DL-lactide-co-glycolide) (PLGA) microspheres (89). These formulations were tested in mice as well as in guinea pigs by comparison with the efficacy and toxicity induced by the naked DNA preparation or BCG.

The single-shot prime-boost formulation clearly presented good efficacy and diminished lung pathology in both mice and guinea pigs (89).

Sugawara and his colleagues compared the protective efficacy of recombinant (Ag85A) BCG Tokyo with Ag85A peptide boosting against MTB-infected guinea pigs in comparison with that of DNA vaccine encoding Ag85A. They concluded that peptide boosting is important for the induction of higher protective efficacy by recombinant BCG Tokyo or a TB DNA vaccine and that both recombinant BCG Tokyo (Ag85A) and (somewhat unexpectedly) Ag85A DNA vaccine induced significant Th2 cytokine mRNA expression (90).

Non-human Primates

As already mentioned earlier, in vivo electroporation has been described to increase humoral immune responses induced by DNA vaccines in rhesus macaques (42). More recently, Okada et al. reported on an evaluation of a novel vaccine (HVJ-liposome/HSP65 DNA (MTB) + human IL-12 DNA) against TB using the cynomolgus monkey model of TB (91). This novel vaccine provided a higher level of protective efficacy than BCG based upon the assessment of mortality, the Erythrocyte Sedimentation Rate (ESR), body weight, chest X-ray findings, and immune responses. Furthermore, a prime-boost immunization of HSP65+IL-12/HVJ and BCG showed a synergistic effect in the TB-infected cynomolgus monkey (100 per cent survival). As a caveat, it should be mentioned that the BCG strain used in the Okada study was BCG Tokyo, a strain that has scored weakly in some comparative studies.

Conclusion

In 2006, WHO published its official Global Plan to Stop TB 2006–2015, highlighting 10 Actions that are key to the success of this Plan. It is heartwarming to see that besides diagnostics and drugs a place was reserved for new TB vaccines (92).

There are a few TB vaccine candidates (unfortunately no DNA vaccines) which have recently passed the preclinical testing stage and for which Phase I and Phase II trials have actually started (93). Most advanced are probably the studies by the Oxford group that attempt to increase the efficacy of the existing BCG vaccine by boosting with recombinant MVA expressing Ag85A (94). Two fusion protein-subunit vaccines in adjuvant, that is, the Hybrid1 fusion protein (Ag85B-ESAT-6) (95) and the 72F fusion protein (MTB 32 + MTB39) (96), have also progressed to the clinic. Finally, Phase I trials have started with a recombinant BCG vaccine developed by Marcus Horwitz and his colleagues that overexpresses the Ag85B antigen (97, 98), and with a recombinant BCG

vaccine developed by Stefan Kaufmann and colleagues that secretes listeriolysin, in an attempt to generate stronger MHC class I responses (99). Some excellent reviews on the preclinical testing of new vaccines for TB were written recently (100, 101) and expounded in selected chapters of this book.

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