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Review Article

RECENT DEVELOPMENTS IN THERAPEUTIC DNA VACCINES AGAINST TUBERCULOSIS

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ABSTRACT

DNA vaccines are simple rings of DNA containing a gene encoding an antigen, and a promoter/terminator to express the gene in mammalian cells. Some DNA vaccines which previously showed to induce protective immunity against infection by Mycobacterium tuberculosis (M. TB) in a prophylactic manner are also surprisingly effective when used therapeutically, including persistent Mycobacterium tuberculosis and multidrug-resistant tuberculosis (MDR-TB) which are refractory to immune system and antibacterial chemotherapy alone. When used in combination with antibacterial drugs, therapeutic DNA vaccines could effectively eliminate residual bacteria in infected animals and shorten the therapy course of conventional chemotherapy. Detailed studies demonstrated that therapeutic effects of DNA vaccines may at least partly be due to the restoration of the Th1/Th2 balance. Some problems have also emerged along with these exciting results. Therapeutic DNA vaccine is a promising strategy against tuberculosis, however developing an ideal DNA vaccine for therapy of tuberculosis will require further development. The purpose of this paper is to review recent developments in therapeutic DNA vaccines against tuberculosis.

KEY WORDS: MDR-TB, *Mycobacterium bovis, Mycobacterium tuberculosis,* therapeutic DNA vaccine

INTRODUCTION

Mycobacterium tuberculosis (M.TB) infects about one-third of the world's population, causing approximately 3 million deaths annually (Dye *et a.,l* 1999). Tuberculosis usually attacks the lungs, while sometimes it also attacks other organs of human body such as bone, ovary and stomach. The main distribution of tuberculosis is located in many Asian and African countries. The recent increase in the incidence of TB, particularly antibiotic-resistant tuberculosis underscores the need for an effective vaccine against this lethal disease. The only vaccine currently in use is the live, attenuated strain of *Mycobacterium bovis, Bacille Calmette-Guérin* (BCG), that was derived in the early 1920s (Norazmi and Mustaffa). Although vaccination with BCG is widely practiced worldwide, the efficacy of this vaccine varies from 0% to 85% in different populations. The efficacy of BCG vaccination reduces over a period of 10 to 15 years and there is no or very low prevention against pulmonary TB in adult population (Norazmi and Mustaffa, 2004). As a result, there is an urgent need to develop better or improved TB vaccines as an alternative to BCG. To date, over 170 TB vaccine candidates have been tested in animal models of these represent diverse types including DNA vaccines, sub-units, polyproteins, recombinant BCGs, and live attenuated vaccines. Most have been investigated in mice for prophylactic use. But currently, one third of population in the world has been infected with *M. tuberculosis*, and a considerable public health burden from TB is associated with reactivation of latent infection, which poses an immense problem for current vaccines and vaccination strategies (Dye *et al.,* 1999).

DNA vaccine is an example of non-living candidate vaccines which can be a better approach in vaccine development. DNA vaccine is constructed by inserting the most promising antigens under strong promoters in an expression vector and injected into muscle cells (LIJun-mining and ZHDao-yin, 2006). DNA vaccination has been shown to induce protective cellular and humoral responses in animal models of tuberculosis and also has been shown to give long lasting protection. Previously, researchers had constructed a DNA vaccine encoding the genes for *M. tuberculosis* secreted proteins MPT64 (23kDa), Ag85B (30kDa) and ESAT-6 (6 kDa) as candidate antigens. The result showed that the most effective secreted protein was Ag85B followed by ESAT-6 and then MPT64 (Peng *et al.,* 2005). The protein of the Ag85 complex is a major secretion product of *M. tuberculosis* and *M. bovis* and both Ag85A and Ag85B elicit T-cell responses in TB patients. The Ag85 was tested as a DNA vaccine in animal models and shown to induce high cellular response and confer protection in mice and guinea pig models of TB. Recent studies had shown that one of the immune-reactive antigens against T-cell is 8.4kDa protein. This antigen had showed potential to elicit strong Th-1 cell responses (Luis *et al*., 2008). Some investigators have recently argued that developing vaccines to boost appropriate protective immune mechanisms in individuals who have already been infected may be a promising strategy.

DNA vaccines

An exciting area of the vaccine field in general is the observation that microbial DNA sequences can be used as vaccine targets (Qin *et al*., 1997). The basic strategy is to identify an immunogenic protein, then isolate the gene and insert it into an expression plasmid that possesses a strong promoter gene, use this to transform bacterial cells so as to expand the plasmid, and then isolate it, and use it as a vaccine. Once injected into animal-muscle cells, the plasmid is transcribed to RNA, and the cell expresses the protein (LIJun-mining and ZHDao-yin, 2006). This approach avoids the need for adjuvants, which are problematic in terms of other types of TB-vaccine candidates, and because a living mycobacterium is not involved, it can be safely put into an immunocompromised host (Qin *et al*., 1997 and Peng *et al.,* 2005). DNA vaccines seem to work well because they induce a type of pan-immune response; whatever the response of choice, DNA seems to induce them. After presentation by dendritic macrophages present in the muscle mass, a strong Th1 response can be observed, as well as strong CD8 CTL responses. It is interesting that if given by “gene gun”, which drives particles or liquid droplets into the dermis, Th2 responses are favored. In addition, certain promoter systems, may favor Th1 immunity and resulting protection in mice against TB infection (Qin *et al*., 1997). The two most effective vaccines are against Ag85A and hsp60, although the results regarding the latter have unfortunately become controversial (Coler *et al*., 1998). In terms of the Ag85A vaccine, it reduces the day-30 bacterial load in mice. In guinea pigs, the load is appreciably not changed, but there is excellent, long term survival, and the necrotic lung pathology seen in controls is completely prevented**.** However, as a post-exposure vaccine in mice already infected with *M. tuberculosis*, the Ag85A DNA has no protective effect (LIJun-mining and ZHDao-yin, 2006)**.** In mice given the hsp60 DNA (derived from *M. leprae*), the vaccine is equally, highly effective if given before or during TB infection and in both models, causes a massive reduction in the bacterial load (Shedlock and Weiner, 200).

Emergence and status of DNA vaccines for therapy of TB

There are two targets for a therapeutic TB vaccine: a post-exposure vaccine to prevent the development of disease by boosting protective immune responses in those who are infected with *M. tuberculosis*, and a therapeutic vaccine to enhance the effects of conventional chemotherapy, especially in multidrug resistance *tuberculosis* (MDR-TB) (Luis *et al*., 2008). Therapeutic vaccine is not a novel strategy in the TB research field; Robert Koch had tried such a strategy in the 1890s. However, the vaccine therapy was abandoned because of side effects, and practically no studies directed at a therapeutic vaccine against TB appeared for a long time thereafter. Recently, trials using a subunit vaccine for therapy of TB also suggested that great caution needs to be paid in such strategies (Moreira *et al*., 2002). Coler *et al*., 1998, demonstrated that plasmid DNA injection could elicit an immune response in animals, which opened a new era in vaccinology. Further studies found that DNA vaccines could stimulate both the exogenous and endogenous antigen presentation pathway, and could generate not only specific Th1 cell responses but also CD8 + T cell mediated cytotoxicity. These findings suggest that DNA vaccination may be a promising strategy against intracellular pathogens such as viruses,

some parasites and bacteria that are refractory to the immune system and chemotherapeutic drugs alone (Helen *et al*., 2003).

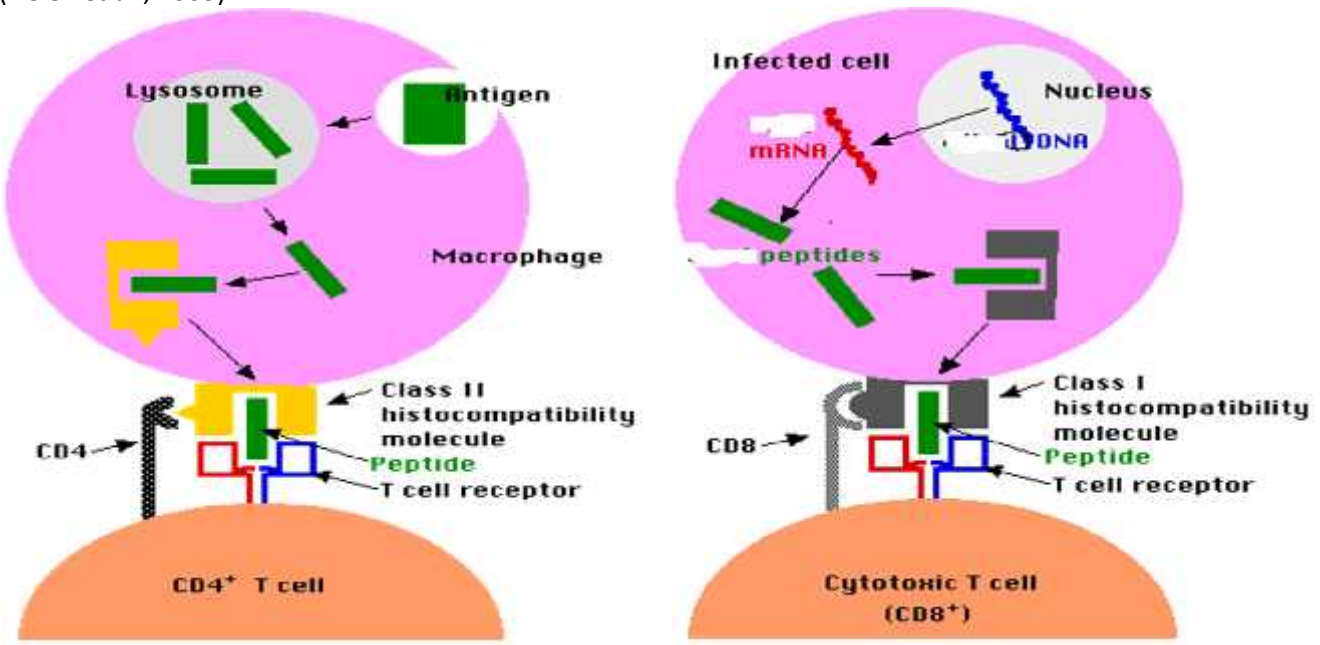

Fig1. Exogenous and endogenous antigen presentation path way (Adapted from Wikipedia)

The value of DNA vaccines against tuberculosis was documented. Subsequently, a number of DNA vaccines encoding various mycobacterial antigens were reported to be protective in prophylactic models. After four intramuscular doses of the plasmid DNA expressing *M. leprae* HSP65, bacterial loads in spleen and lungs of infected mice declined significantly 2 months and 5 months after the first dose of DNA vaccination (Norazmi and Mustaffa, 2004). Plasmid DNA expressing *M. leprae* HSP70 and *M. tuberculosis* ESAT-6 were also effective in reducing the bacterial load in mice infected with *M. tuberculosis* (Norazmi and Mustaffa, 2004). Furthermore, a DNA vaccine was found to be a good adjuvant to antibacterial chemotherapy. Given after chemotherapy, three intramuscular doses of HSP65 DNA vaccine appeared to eliminate residual bacteria from mice (Norazmi and Mustaffa, 2004 and Peng *et al.,* 2005). Both Ag85A and IL-12 DNA vaccines were effective in preventing reactivation of TB in mice when simultaneously used with conventional antibacterial chemotherapy. However subsequent results from other laboratories using various DNA vaccines in infected murine models produced inconsistent results (Flynn *et al.,* 1995). When plasmid V1Jns containing a gene encoding Ag85A of *M. tuberculosis* (which had been shown to induce protective immunity in mice) was injected into mice previously infected with *M. tuberculosis*, a significant reduction in splenic bacterial load was found, but no bacterial and histological changes were detected in the lungs (Moreira *et al.,* 2002). Later, with a view to test whether immunization with TB DNA vaccine would retard the endogenous reactivation of the latent bacilli and exogenous reinjection, Repique and colleagues injected a TB DNA vaccine combination (consisting of 10 different single DNA vaccines) into infected mice after a course of drug treatment. This DNA vaccine combination offered only a modest decrease in the growth of a secondary *M. tuberculosis* challenge, but did not prevent recrudescence of the infection (Peng *et al.,* 2005). DNA vaccine encoding HSP65 of *M. leprae*, which had been reported to have both prophylactic and therapeutic protective effects against intravenous infection of mice with *M. tuberculosis*, could induce severe pathogenic reactions in mice, characterized by multifocal discrete regions of cellular necrosis throughout the lung (Coler *et al*., 1998). Similar necrosis was also seen in mice immunized with a DNA vaccine encoding the Ag85 antigen of *M. tuberculosis*. Such an immune-pathological reaction (known as a Koch reaction) after DNA vaccination posed a significant safety problem to the development of therapeutic DNA vaccines (Moreira *et al.,* 2002). Fortunately, this enhanced immunopathologic reaction after DNA vaccination has not been observed in other studies, and more recently studies have provided more promising results. A report by Kernodle demonstrated that a DNA vaccine encoding *M. leprae* HSP65 induced a significant reduction of bacterial loads immediately after the first month

of treatment when used with combination antibacterial chemotherapy (Kernodle, 2010). Bacilli in the lungs of the animals could not be detected at 6 months after the beginning of this treatment. Moreover, this treatment was found to be effective against both *M. tuberculosis* H37Rv and MDR-TB infection, which indicated that effective therapeutic DNA vaccines could potentially overcome the limitations of chemotherapy against MDR-TB (Shedlock and Weiner, 200).

DNA vaccines could also retard the reactivation of latent bacteria in mice and shorten the period of conventional chemotherapy when combined with conventional antibacterial drugs. Recent studies found that Ag85B DNA vaccine could significantly decrease the pulmonary and splenic bacterial loads and reduce pulmonary lesions in mice infected with virulent *M. tuberculosis* (Kernodle, 2010).

Mechanisms of therapeutic DNA vaccines

In studies directed at elucidating host immune mechanisms in response to *M. tuberculosis*, researchers recognized that the protective effects against TB mainly depend on the Th1 cell response, which involves immune responses mediated by both CD4 + and CD8 + T cells. However progressive TB is usually associated with a Th2 cell response, which suggested that *M. tuberculosis* could block the hosts' protective immunity (LIJun-mining and ZHDao-yin, 2006). In an attempt to enhance the therapeutic effects of DNA vaccines, some studies tried to enhance the immunogenicity of vaccines to Th1 cells adopted a multigene DNA vaccination strategy by co-immunization with 10 different DNA vaccines (Kernodle, 2010). Protection against TB is not directly associated with the magnitude of the Th1 response. The potentially protective Th1 response may be converted to an immune -pathological response when a Th2 response is superimposed on that Th1 response. To block this immune-pathology, successful immunotherapy needs to arrest this subversion, and towards that end, DNA vaccines may be a promising option (Repique *et al.,* 2002). Studies both in prophylactic and therapeutic mode demonstrated that DNA vaccination could significantly decrease the frequency of IL-4 producing T cells and increase the frequency of IFN-γ producing T cells in mice (Flynn *et al.,* 1995). This switch from Th2 to Th1 paralleled a substantial reduction of pathology in the animal and these results were further supported by recent results. Taken together, these studies indicated that the therapeutic effect of DNA vaccines may at least partly be due to the rectification of immunopathological subversion and the restoration of the Th1/Th2 balance (Flynn *et al.,* 1995). It is becoming clear that cytosine phosphate guanosine (CpG) DNA and synthetic oligodeoxynucleotides containing unmethy- lated CpG are potent inducers of components of the innate immune system including dendritic cells (DCs), macrophages, natural killer (NK), and NKT cells. Because it can strongly induce IL-12 and IFN-γ, CpG DNA can also direct the immune response to a Th1 phenotype (Condon *et al*., 1996). Consistent with this, modest protective effects and a Th2 to Th1 switch were also observed after the vaccination of empty plasmid DNA in some studies. However, the protective efficacy was significantly elevated when mycobacterial antigens were expressed by the DNA vaccines. It therefore appears likely that the therapeutic effects of DNA vaccine result from a combined effect of innate immunity driven by CpG motifs in the plasmid backbone together with mycobacterial antigen- specific immunity (Okaqda *et al*., 2011). In gene immunization, the bacteria gene of interest is converted to DNA, which is inserted in a bacterial plasmid. The DNA plasmids carrying one or several genes or several different plasmids each carrying one or several genes can be administered in the skin or at the mucosa. The same gene(s) can be introduced in a bacterial vector and used either as the only vaccine or as a boosting component to the first DNA vaccination (Repique *et al.,* 2002). The plasmid enters the cell nucleus, where the gene initiates transcription, followed by protein production in the cytoplasm. Secreted proteins induce cytokines, T help, and Abs that will react with and eliminate M.TB. APCs present peptides in context of the MHC of the vaccinated individual and activate cytokines and killer cells, which in turn will lyse infected cells. DNA itself or cytokines in the immune cascade activate NK cells. In prophylactic vaccination, naïve B and T cells are primed by proteins and by APC presenting peptides, respectively. In therapeutic vaccination, the antigens may provide both priming of new responses (Ulmer *et al*., 2006).

TB vaccine strategies

A. Pre-infection vaccine strategy: from a public health perspective, delivering a vaccine prior to *mycobacterial* infection and soon after birth makes most sense.

B. Booster vaccination strategyW would be to use a new TB vaccine as a booster sometime after neonatal BCG vaccination
C. Post-infection vaccine strategy: is to prevent disease by enhancing or boosting immunity in persons already infected a post-infection vaccine strategy. This approach is attractive because more than 2 billion persons worldwide are already infected and therefore at risk of progression to disease.
D. Therapeutic vaccine: a fourth option would be to use a vaccine as an adjunct to anti-TB treatment, to shorten therapy or reduce the risk of relapse, a therapeutic vaccine. This may be particularly relevant in situations where multi-drug resistant TB cases are common.

Development of more-effective preventative vaccines

It has been well established that Th1 responses are required for control of mycobacterial infection in both mice and humans. Thus, due to the potency of DNA and CpG ODNs in Th1 responses, it would follow that DNA would have a beneficial role in *M. tuberculosis* infection. First, with regard to vaccination, DNA vaccines encoding antigens including 85A and HSP have been shown to reduce the mycobacterial load following infectious challenge in mice (Huyggen *et al*., 1996). In some of the studies, however, the protection achieved by DNA vaccination was not any more effective than that in mice vaccinated with BCG alone. Thus, due to the longstanding safety profile in humans, a study was done to try to improve the efficacy of BCG vaccination. In this regard, it was demonstrated that mice vaccinated with BCG plus IL-12 protein or CpG ODNs had a reduction in mycobacterial load from the lungs following an aerosol challenge with a virulent strain of *M. tuberculosis* (Freidage *et al*., 2000). Because other models of intracellular infection (e.g. malaria and HIV) have shown that a prime-boost approach with DNA vaccines can enhance immunity and efficacy, a similar approach — involving DNA, BCG with adjuvants, or even recombinant vaccinia virus encoding *M. tuberculosis*-specific antigen—could also enhance protective efficacy in *M. tuberculosis* infection (Norazmi and Mustaffa, 2004).

Therapeutic vaccines to reduce the duration of therapy

Another major issue is the optimization of treatment for existing disease. Currently, treatment regimens for tuberculosis require that patients take a combination of antibiotics for at least six months. Although this regimen is effective, compliance is often poor and this results in incomplete treatment and the development of drug-resistant strains. Thus, the development of a treatment regimen in which the duration of antibiotic treatment is reduced and clinical effectiveness is maintained may increase patient compliance and reduce potential for drug-resistant strains (Flynn *et al.,* 1995).

Recently immunotherapy (vaccination with DNA encoding HSP65) following standard antimycobacterial chemotherapy resulted in more complete eradication of an intravenous *M. tuberculosis* infection than chemotherapy alone (Lowie *et al*., 1996). Vaccination with DNA expressing *M. tuberculosis* antigen or IL-12 was effective in reducing the bacterial load of an already established systemic *M. tuberculosis* infection. Although these studies provided an elegant proof-of-concept for DNA vaccination as immunotherapy against tuberculosis, it remains to be determined whether DNA is effective either alone or combined with chemotherapy against a natural aerosol infection. Thus, based on all of these data, DNA vaccines and CpG ODNs should be able to play a role as both therapeutic and prophylactic vaccines against *M. tuberculosis* (Lowie *et al*., 1996).

Problems for the future (challenges)

DNA vaccines have many potential advantages; they are easily synthesized, stable, and safe for the immune-compromised host. Most importantly DNA vaccines can efficiently induce both CD4 + and CD8 + T cell responses, and can switch the immune response from one that is relatively inefficient and produces only bacteria stasis to one that kills bacteria (Condon *et al*., 1996). But a lot of uncertainty regarding of DNA vaccines exists when they were used in therapeutic mode. Why do some DNA vaccines that effectively protect naïve animals against *M. tuberculosis* infection have no protective effect in post exposure models? Why do DNA vaccines used in therapeutic models have different or even opposite effects in different laboratories? These discrepancies may be due to the complex nature of stimulated immune cells in the host, and may be related to the specific animal species, bacterial strains of infection, and even the routes and dose of infection (Ulmer *et al*., 2006). To date, we do not have a satisfactory animal model that mimics TB development in humans. Therefore, even if a vaccine against TB proves effective in animal models, we have no way of knowing

whether the data obtained has clinical relevance. This may represent the single greatest obstacle to develop and evaluate a vaccine against TB (Flynn *et al.,* 1995 and Moreira *et al.,* 2002). Another important but uncertain factor impacting the effect of DNA vaccine is the choice of the target antigen. To date, most DNA vaccines targeted immune-dominant proteins released into the filtrate from cultures of *M. tuberculosis* and heat shock proteins of mycobacteria (Luis *et al*., 2008). But some investigators have questioned whether adding to an already potent immune response may be detrimental, since in the postexposure vaccination model, the immune system has already recognized and responded to these immnodominant proteins. However, some of these antigens did show therapeutic effects in mice. Clarifying mechanisms of therapeutic DNA vaccines is therefore an urgent task for future investigations (Luis *et al*., 2008 and Fuller *et al*., 2006). Other factors such as the vaccination route, the frequency of vaccination, and the time of observation may also impact the therapeutic and prphylactic effects of DNA vaccines. However, we do not yet have a standard protocol in vaccination studies. Also, we cannot exclude the possibility that the DNA would integrate into the host genome or whether it might cause autoimmune disease. All these uncertainties may also inhibit develop and evaluation of DNA vaccines against TB (Fuller *et al*., 2006 and Lori et al., 2007). Aside from these obstacles, great efforts have produced encouraging progress in this field. With the completion of the *M. tuberculosis* H37Rv genome sequencing project and the development of downstream sciences such as comparative genomics, transcriptomics and proteomics, great advances have been made in identifying promising target antigens (Coler *et al*., 1998). Some strategies (such as prime-boost regimens) to improve the relatively poor immunogenicity of DNA vaccines that involve enhancing the level of immune response to a target antigen have also been tested and have produced very promising results [(Helen *et al*., 2003 and Zaharoff *et al*., 2002).

Considerable work will be required to develop an ideal DNA vaccine for the therapy of TB. But because of its potential advantages to public health, developing DNA vaccines against TB is a worthy goal that deserves serious attention (Fuller *et al*., 2006).

CONCLUSION

Nucleic acid vaccines are good candidates for the prophylaxis of intracellular pathogens like mycobacterium due to the Th1 response mediated by CD4+ and CD8+ that they induce. TB DNA vaccines for both therapeutic and protective purposes have proven to restore the Th1/ Th2 balance, resulting in a significant reduction of the pathology in the animal. Different antigens given alone in recombinant and DNA vaccines have been under investigation, but to date, no vaccine alone tested in clinical trials has proven to be more effective against Mycobacterium than BCG. The Ag85 family has by far been the most studied encoded antigen for TB vaccines. Studies have shown that both Ag85A and Ag85B can induce a robust Th1 immune response with elevated IFN-γ, IL-2 and TNF-α. Other targeted antigens were Rv3407 Hsp65, ESAT-6 and MPT64/MPT83 among others. Most of these vaccine schedules consisted of priming with BCG and boosting with the specific DNA vaccine in order to achieve a superior response compared with BCG alone. A better understanding of the molecular mechanisms that obstruct the immune response in TB may aid in the identification of novel molecular targets that can be blocked in order to enhance the therapeutic effect of TB DNA vaccines. With continued endeavor in the development of TB therapeutic vaccines, we can foresee that TB therapeutic DNA vaccines will emerge as a significant approach.

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