In silico Analysis and Experimental Validation of *Mycobacterium tuberculosis*-Specific Proteins and Peptides of *Mycobacterium tuberculosis* for Immunological Diagnosis and Vaccine Development

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Key Words

In silico analysis · *Mycobacterium tuberculosis* · Peptides · Proteins · Diagnosis · Vaccine

Abstract

Comparative analyses of the *Mycobacterium tuberculosis* genome with the genomes of other mycobacteria have led to the identification of several genomic regions of difference (RDs) between *M. tuberculosis* and *M. bovis* BCG. The identification of immunodominant and HLA-promiscuous antigens and peptides encoded by these RDs could be useful for diagnosis and the development of new vaccines against tuberculosis. The analysis of RD proteins and peptides by in silico methods (using computational programs to predict major and HLA-promiscuous antigenic proteins and peptides) and experimental validations (using peripheral blood mononuclear cells and sera from tuberculosis patients and BCG-vaccinated healthy subjects to assess antigen-specific cellular and humoral immune responses in vitro) identified several major antigens and peptides. To evaluate the in vivo potentials, the genes of immunodominant antigens were cloned and expressed in DNA vaccine vectors. Immunizations of experimental animals with the recombinant constructs induced antigen-specific cellular responses. Further experiments showed that each of these proteins had several T and B cell epitopes scattered throughout their sequence, which confirmed their strong immunogenicity. In conclu-

sion, the bioinformatics-based in silico identification of promiscuous antigens and peptides of *M. tuberculosis* is a useful approach to identify new candidates important for diagnosis and vaccine applications.

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Introduction

Tuberculosis (TB) is a major infectious disease problem of worldwide prevalence and ranks among the top 10 causes of global mortality. The most recent estimates from the WHO suggest that there are 8.7 million new cases of TB (range 8.3–9.0 million) globally, equivalent to 125 cases per 100,000 individuals, and 1.4 million people died from TB in 2011, including almost one million deaths among HIV-negative individuals and 430,000 among people who were coinfecting with HIV [1]. Furthermore, it is estimated that one third of the world's population is latently infected with *Mycobacterium tuberculosis* and about 10% of these people will develop active disease in their lifetime. In spite of worldwide efforts to control TB, the global burden of the disease is worsening in the poor developing countries of Asia and Africa. This is due to many reasons, including wars and immigration, poverty and malnutrition, HIV-TB coinfection, and the increasing problem of multidrug-resistant and excessive drug-resistant TB, etc. [1]. The worldwide control of TB

requires an effective control and eradication strategy using cost-effective methods/reagents for specific diagnosis and prophylactic and/or immunotherapeutic vaccine(s) that can be given safely [2, 3]. In particular, effective vaccines are considered the best weapons to fight against infectious diseases [2].

Tuberculin Skin Test for the Diagnosis of TB

Currently, the tuberculin skin test (TST) is the only test available globally for the in vivo immunological diagnosis of TB. The antigenic preparation in TST is the purified protein derivative (PPD) of *M. tuberculosis*, which is a crude mixture of molecules present in the culture filtrate of in vitro grown *M. tuberculosis*. Hence, the PPD contains substances that are *M. tuberculosis* specific as well cross-reactive with other mycobacteria. Although it is a simple and cost-effective test, TST results must be interpreted carefully because a negative test does not rule out a diagnosis of TB but may reflect the presence of nonresponsiveness due to the immunocompromised state of the patients or incorrect test procedures [4]. In addition, a positive TST cannot distinguish between active disease, latent infection with *M. tuberculosis*, BCG vaccination, or cross-sensitization by environmental mycobacteria [4]. Thus, the TST has poor diagnostic value, especially in geographic areas and countries where BCG is routinely used, the prevalence of TB is low, or the environmental burden of nontuberculous environmental mycobacteria is high [3–6]. Therefore, a TB-specific skin test requires the development of new tuberculin(s) consisting of antigens specific for *M. tuberculosis*.

***M. bovis* BCG as a Vaccine against TB**

The currently available vaccine against TB is BCG, which is a live attenuated strain of *M. bovis*. Although it has been widely used to vaccinate against TB for about 90 years, BCG is the most controversial vaccine being used in humans. This is because BCG has failed to protect against TB in different parts of the world, especially in adults with pulmonary TB [3, 7]. The variations in protection have ranged from nil (e.g. in India and Malawi) to 80% (e.g. in the UK) against pulmonary TB in adults [3, 8]. Furthermore, BCG is not suitable for vaccination of immunocompromised individuals, particularly HIV/AIDS patients, due to the fear of causing disease in such individuals [9]. Moreover, because BCG vaccination in-

duces a positive skin test response to PPD, it becomes difficult to use TST for diagnostic or epidemiological investigations in BCG-vaccinated populations [3]. Therefore, the development of new vaccines based on *M. tuberculosis*-specific antigens is urgently needed.

Comparative Genomics to Identify *M. tuberculosis*-Specific Genomic Regions Deleted/Absent in *M. bovis* BCG and Approaches to Obtain Regions of Difference Proteins or Peptide Equivalents for Immunological Evaluation

The identification of *M. tuberculosis*-specific antigens has been facilitated by the relatively recent advances in genomics of mycobacteria, leading to the sequencing of complete genomes of various mycobacterial species, including *M. tuberculosis*, *M. bovis*, and BCG, etc. Comparative analyses of mycobacterial genomes have identified 16 genomic regions of *M. tuberculosis*, which are deleted/absent in one or more strains of BCG [10, 11]. Among these regions of difference (RDs), 11 RDs (RD1, RD4 to RD7, RD9 to RD13, and RD15) of *M. tuberculosis* H37Rv are absent in all BCG substrains currently used as vaccines to protect against TB in different parts of the world (table 1). In silico analysis suggested that these RDs have 86 open reading frames (ORFs) capable of encoding equal numbers of proteins (table 1), [10]. Further studies have suggested that in silico predictions of RD ORFs could be relevant to protein expression in *M. tuberculosis* because the expression of all of the predicted RD1 genes, at the mRNA level, was confirmed in *M. tuberculosis* using reverse-transcriptase PCR assays [12, 13]. Attempts have been made to obtain RD proteins by using recombinant methods of DNA cloning and expression, followed by purification of recombinantly expressed proteins, or their equivalents using overlapping synthetic peptides covering the sequence of each protein [14–17]. As described below, these recombinant antigens and synthetic peptides have been tested in antibody and cellular assays to identify the candidate RD proteins/peptides suitable for diagnosis and vaccine development.

Purified RD Proteins and Synthetic Peptides for Immunological Reactivity

To characterize RD proteins for immunological reactivity, it is essential that these proteins be obtained in a purified form, free from other cellular proteins of *M. tu-*

Table 1. Some features of 11 RDs of *M. tuberculosis* deleted/absent in all *M. bovis* BCG vaccine strains

RD		Predicted ORFs in each RD		Synthetic peptides to cover the ORFs of each RD, n
designation	size, kb	n	designation	
RD1	9.5	9	Rv3871-Rv3879	220
RD4	1.9	3	Rv0221-Rv0223c	80
RD5	2.8	5	Rv3117-Rv3121	72
RD6	12.8	11	Rv1506c-Rv1516c	236
RD7	9.0	8	Rv2346c-Rv2353c	167
RD9	5.9	7	Rv3617-Rv3623	108
RD10	3.0	3	Rv1255c-Rv1257c	71
RD11	4.9	5	Rv3425-Rv3429	84
RD12	2.0	4	Rv2072c-Rv2075c	83
RD13	11.0	16	Rv2645-Rv2660c	225
RD15	12.7	15	Rv1963c-Rv1977	302
Total peptides				1,648

berculosis. The natural expression of all putative/predicted RD proteins in *M. tuberculosis* could not be determined due to the lack of reagents which are required to obtain purified proteins from *M. tuberculosis* cultures. On the other hand, the DNA sequences of all of the putative/predicted RD proteins were known from the genome sequence [10]; therefore, attempts were made to express them in *Escherichia coli* cells and purify the recombinant mycobacterial proteins in quantities sufficient for immunological assays [18–25]. However, the production of mycobacterial proteins in general and proteins of large size in particular has been quite difficult in *E. coli* for various reasons, including difficulties in amplification of target genes from genomic DNA of *M. tuberculosis*, degradation of the expressed proteins by *E. coli* proteases, and problems in purification of mycobacterial proteins from *E. coli* components [18–24].

To overcome the problems associated with recombinant protein expression and purification, pools of synthetic peptides covering the coding sequence of mycobacterial proteins have been used to faithfully replace full-length proteins in immunological assays [26–35]. Therefore, to test 86 ORFs of 11 RDs of *M. tuberculosis* in immunological assays, a total of 1,648 synthetic peptides were synthesized [36–40], (table 1). All of the peptides were 25-mers and overlapped with the neighboring peptides of the same ORF by 10 residues [41–45]. The purpose of the 10-residue overlap was to greatly reduce the probability of missing the epitopes of individual ORFs, because mycobacterial epitopes recognized in immunological assays are usually <10 aa in length [46].

Identification of Major RD-Encoded Proteins/Peptides Reactive in Antibody Assays

A total of 775 synthetic peptides covering the sequence of 39 ORF proteins encoded by genes predicted in 5 RDs of *M. tuberculosis*, i.e. RD1, RD4, RD5, RD6, and RD7, were tested by enzyme-linked immunosorbent assays for antibody reactivity with sera from HIV-negative pulmonary TB patients (n = 100) and *M. bovis* BCG-vaccinated healthy subjects (n = 100). The results identified 4 immunodominant peptides reactive with TB sera, i.e. aa 346–370 of RD1ORF Rv3876, aa 241–265 of RD6ORF Rv1508c, aa 136–160 of RD6ORF Rv1510, and aa 325–336 of RD6ORF Rv1516c [47]. However, only 3 of them, i.e. aa 346–370 of Rv3876, aa 241–265 of Rv1508c, and aa 325–336 of Rv1516c, had significantly stronger antibody reactivity with sera from TB patients versus healthy subjects ($p < 0.05$), and significantly higher positivity with TB sera (% positives = 66–93%) versus sera from healthy subjects (% positives = 10–28%) [47]. In silico analysis for antibody epitopes using an ABCPred server showed that each of the peptides had multiple epitopes for recognition by antibodies. To verify these predictions, anti-peptide antibodies were raised in rabbits after immunization with pools of 11 peptides corresponding to each protein. The results showed that these peptides could induce production of antibodies in vivo. Probing of culture filtrate and whole cell lysates of *M. tuberculosis* with anti-peptide antibodies suggested the natural expression of Rv3876, Rv1508c, and Rv1516c in whole cell lysates of *M. tuberculosis*. These results suggest that the immunodominant RD peptides with serodiagnostic potential are naturally

Table 2. Summary of the names of the RD1 genes, the ORF annotation, and the proteins

Gene name	ORF annotation	Protein name
<i>Rv3871</i>	Rv3871	Rv3871
<i>pe35</i>	Rv3872	PE35
<i>ppe68</i>	Rv3873	PPE68
<i>esxb/cfp10</i>	Rv3874	ESXB/CFP10
<i>esxa/esat6</i>	Rv3875	ESXA/ESAT-6
<i>Rv3876</i>	Rv3876	Rv3876
<i>Rv3877</i>	Rv3877	Rv3877
<i>Rv3878</i>	Rv3878	Rv3878
<i>Rv3879c</i>	Rv3879	Rv3879

expressed in *M. tuberculosis* [47]. A number of other studies also suggest the diagnostic potential of RD proteins and peptides based on detection of antibodies in sera of TB patients [48–50].

In silico Analysis of RD Proteins for T Cell Epitopes

The presentation of antigens to T cells requires their processing into small peptides by antigen-presenting cells and association with major histocompatibility complex (MHC) molecules. The length of T cell epitopes usually ranges from 8 to 10 aa, which could easily be synthesized using standard chemistries to overcome the problems associated with obtaining full-length recombinant proteins. The use of synthetic peptides in TB has been successfully demonstrated for diagnostic applications in humans and cattle [51, 52], and as a protective vaccine in mice [53]. In most studies, overlapping synthetic peptides covering the entire sequence of a protein have been used to identify the peptides recognized by T cells [54–57]. However, usually a fraction of peptides of large size proteins are recognized by T cells [54–60], and therefore the overlapping peptide approach becomes quite costly and impractical if a large number of proteins are to be tested for T cell reactivity. Since Th1 cells recognize mycobacterial antigens and epitopes in association with MHC class II molecules [46], an alternative approach has been to screen proteins for identification of regions that can associate with MHC molecules [61–64], and then test the peptides predicted to bind the MHC molecules for T cell reactivity. Such an approach drastically reduces the cost to screen for Th1 cell reactivity [65].

Although, several prediction programs have been proposed to identify peptides capable of binding to MHC mol-

ecules [66–68], virtual matrix-based prediction programs TEPITOPE and ProPred have been successfully employed to identify HLA-DR ligands derived from tumors and endogenous proteins involved in autoimmune diseases [69, 70]. In addition, these computer-assisted programs have previously been shown to accelerate research related to the design of vaccines and diagnostic tests through the identification of promiscuous peptides of mycobacterial proteins [71, 72]. These encouraging results have led researchers to investigate the use of the ProPred program to predict the HLA-DR binding sequences in 3 major antigenic proteins of *M. tuberculosis* encoded by RD1 region genes, i.e. PPE68, CFP10, and ESAT6, and to further confirm the prediction results in identification of promiscuous peptides by evaluating the predicted peptides for promiscuous presentation to T cells in humans [73].

The results suggested that some of the promiscuous peptides capable of Th1 cell reactivity in HLA heterogeneous populations can be predicted from the in silico HLA-DR binding analysis of complete proteins [73]. Therefore, a similar analysis was expanded to all 9 ORFs/proteins encoded by RD1 genes (table 2). The ProPred analysis of complete protein sequences of ORFs Rv3871 to Rv3879 was performed to identify HLA-promiscuous proteins for presentation to T cells. The results showed that all of the 9 ORFs of RD1 were promiscuous HLA-DR binders (table 3). The ProPred analysis further showed that several of them, i.e. Rv3871 and Rv3876 to Rv3879c, were predicted to bind all 51 (100%) HLA-DR alleles included in ProPred (table 3). The remaining ORFs, i.e. Rv3873 to Rv3875, were predicted to bind 84–98% of HLA-DR alleles included in ProPred (table 3). Furthermore, peptide pools of all of these ORFs were promiscuously recognized by peripheral blood mononuclear cells of appropriate donors (TB patients/BCG-vaccinated healthy subjects) (table 3). These results suggest that none of the RD1 protein sequences will have limitations in terms of binding to MHC class II molecules for presentation to Th1 cells. Hence, all of these proteins will not be limited due HLA-restriction, because the HLA-DR alleles included in ProPred are among those which are frequently expressed in different populations.

Identification of Major RD-Encoded Proteins Reactive in Cellular Assays

The protective immunity against *M. tuberculosis* is primarily mediated by cellular immunity (CMI) involving the interaction of antigen-specific Th1 cells and macro-

Table 3. HLA-DR binding prediction by ProPred analysis for the RD1 ORF protein sequence of Rv8371 to Rv3879 and their experimental validation in a Th1-cell assay

RD1 sequence	ProPred analysis for HLA-DR binding to										Positive in the Th1 assay, %	
	DRB	1.1	1.3	1.4	1.7	1.8	1.11	1.13	1.15	5.1		all alleles
Alleles predicted to bind/alleles included in ProPred												
Rv3871		2/2	7/7	9/9	2/2	6/6	9/9	11/11	3/3	2/2	51/51 (100%)	53
Rv3872		2/2	6/7	9/9	0/2	3/6	9/9	10/11	2/3	2/2	43/51 (84%)	33
Rv3873		2/2	6/7	9/9	2/2	6/6	9/9	11/11	3/3	2/2	50/51 (98%)	68
Rv3874		2/2	7/7	9/9	0/2	5/6	9/9	10/11	0/3	2/2	44/51 (86%)	80
Rv3875		1/2	7/7	9/9	2/2	3/6	9/9	11/11	1/3	0/2	43/51 (84%)	48
Rv3976		2/2	7/7	9/9	2/2	6/6	9/9	11/11	3/3	2/2	51/51 (100%)	50
Rv3877		2/2	7/7	9/9	2/2	6/6	9/9	11/11	3/3	2/2	51/51 (100%)	33
Rv3978		2/2	7/7	9/9	2/2	5/6	9/9	11/11	2/3	2/2	49/51 (100%)	40
Rv3879c		2/2	7/7	9/9	2/2	6/6	9/9	11/11	3/3	2/2	51/51 (100%)	ND
MPT83P2 ^a		1/2	0/7	0/9	0/2	1/6	0/9	1/11	0/3	0/2	3/51 (6%)	6
MPT83P14 ^b		2/2	7/7	9/9	2/2	6/6	9/9	11/11	1/3	0/2	47/51 (92%)	65

ND = Not determined. ^a MPT83P2 nonpromiscuous peptide used as a negative control [60]. ^b MPT83P14 promiscuous peptide used as a positive control [60].

phages [74–78]. This interaction is often indicated by antigen-induced proliferation of T cells and is dependent on the interplay of cytokines secreted by these cells [79–81]. Although, a broad spectrum of cytokines may contribute to protection, the T helper type 1 (Th1) cytokines, dominated by interleukin (IL)-2 (responsible for proliferation of antigen-reactive T cells) and IFN- γ secretion, are considered principal mediators of protective immunity against TB [82–84]. Therefore, Th1 cell reactivity, indicated by antigen-induced cell proliferation and preferential secretion of IFN- γ has been used to identify antigens involved in protective immunity [85–90]. Hence, it was considered appropriate to test the proteins encoded by the RD genes of *M. tuberculosis* for these in vitro correlates of protective immunity to identify new candidates of RD-derived subunit vaccines against TB.

About a decade back, both recombinant proteins and overlapping synthetic peptides, corresponding to selected antigens of *M. tuberculosis*-specific RD1, were tested to identify antigens reactive in protective Th1 cell assays [91–94]. However, because of the ease and feasibility of getting the required peptides synthesized, recent studies have used 1,648 overlapping synthetic peptides covering all 86 ORFs predicted in 11 RDs deleted/absent in BCG (table 1). In these studies, CMI reactivities in response to peptide pools of each of the 11 RDs have been determined in relation to protective Th1-type responses by evaluating antigen-induced proliferation and secretion of IFN- γ using periph-

eral blood mononuclear cells obtained from pulmonary TB patients and healthy humans. The results showed that the highest Th1-responses were induced by peptide pools of RD1, RD7, and RD9 peptides [15, 16, 36, 37].

To identify individual proteins of Th1 cell-reactive RDs, further testing of the peptide pools corresponding to each protein of RD1, RD7, and RD9 has also been performed in Th1 cell assays. The results showed that 3 proteins of RD1, i.e. PPE68, ESAT6, and CFP10, and 2 proteins of RD7 (Rv2346c and Rv2947c) and RD9 (Rv3619 and Rv3620) were the best stimulators of Th1 cells in antigen-induced proliferation and/or IFN- γ secretion assays [41, 42, 95–97]. Among these proteins, ESAT6 and CFP10 were identified several years back as strong Th1-stimulating antigens using classical methods of protein purification from culture-filtrate of *M. tuberculosis*, and have been shown to have vaccine potential in animal models of TB [98–100]. However, ESAT6 and CFP10 are also antigens recommended for the specific diagnosis of active and latent TB [101–103] and are widely used for this purpose. Therefore, these antigens cannot be used as vaccines against TB. Hence, other Th1-stimulating antigens of RDs, i.e. PPE68, Rv2346c, Rv2947c, Rv3619, and Rv3620, must be investigated for protective efficacy in animals. If found promising in such studies, these antigens may be further explored in clinical trials as new-generation or alternative subunit vaccines against TB in humans.

Relevance of RD Proteins in Diagnosis Using Delayed-Type Hypersensitivity Responses in Guinea Pigs

The diagnostic potentials of 4 antigens encoded by *M. tuberculosis*-specific RD1 region genes (PE35, PPE68, CFP10, and ESAT-6) and RD9 region gene Rv3619c were further investigated for delayed-type hypersensitivity (DTH) skin responses in guinea pigs [104]. The recombinant *M. tuberculosis* proteins were expressed in *E. coli* and purified to homogeneity by affinity chromatography. Groups of guinea pigs were injected with heat-killed *M. tuberculosis* and live BCG, *M. avium*, and *M. vaccae*. Two to 4 weeks later, the guinea pigs were challenged intradermally in the flank region with mycobacterial sonicates and purified recombinant proteins. The DTH responses were quantitated by measuring erythema at the sites of injections after 24 h. The results showed that all mycobacterial sonicates induced positive DTH skin responses in *M. tuberculosis*-, *M. bovis* BCG-, *M. avium*-, and *M. vaccae*-injected guinea pigs. The purified proteins PE35, PPE68, CFP10, and ESAT-6 elicited positive DTH skin responses in the *M. tuberculosis*-injected group but not in BCG-, *M. avium*-, and *M. vaccae*-injected guinea pigs, whereas Rv3619c elicited positive DTH skin responses in *M. tuberculosis* and in BCG-injected groups but not in *M. avium*- and *M. vaccae*-injected guinea pigs. The overall results showed that the recombinant RD1 antigens induced *M. tuberculosis*-specific DTH skin responses [104–106]. Thus, these antigens may be useful in the specific diagnosis of tuberculosis and therefore could provide an alternative to the currently used PPD-based DTH skin test.

Expression of RD Antigens in DNA Vaccine Vehicles and Immunological Evaluation of Recombinant Plasmids

RD1 PE35, PPE68, *EsxA*, *EsxB*, and RD9 *EsxV* genes were cloned into DNA vaccine vectors capable of expressing them in eukaryotic cells as fusion proteins, fused with immunostimulatory signal peptides of human IL-2 (hIL-2) and tissue plasminogen activator (tPA). The recombinant DNA vaccine constructs were evaluated for induction of antigen-specific cellular immune responses in mice. In brief, DNA corresponding to the above RD1 and RD9 genes were cloned into DNA vaccine plasmid vectors pUMVC6 and pUMVC7 (with hIL-2 and tPA signal peptides, respectively), and a total of 10 recombinant

DNA vaccine constructs were obtained. BALB/c mice were immunized with the parent and recombinant plasmids and their spleen cells were tested for antigen-induced proliferation with antigens of *M. tuberculosis* and pure proteins corresponding to the cloned genes. The results showed that antigen-specific proliferation responses were observed for a given antigen only with spleen cells of mice immunized with the homologous recombinant DNA vaccine construct. The mice immunized with the parent plasmids did not show positive immune responses to any of the antigens of the cloned genes [107]. Mice immunized with the recombinant plasmid DNA (pUMVC6/PE35) showed positive Th1-type cellular responses to pure PE35, but not to an irrelevant antigen, i.e. PPE68 (Rv3873). However, the vaccine construct did not induce antigen-specific Th2-type (IL-5) or anti-inflammatory (IL-10) reactivity to PE35. Testing with synthetic peptides showed that Th1-type cells recognizing various epitopes of PE35 were induced in mice immunized with pUMVC6/PE35 DNA [108]. In another study, recombinant BCG expressing PE35 also induced Th1 responses in mice [109]. These results suggest that pUMVC6/PE35 may be useful as a safer vaccine candidate against TB.

Conclusion

Bioinformatics-based in silico methods for identification of expressed proteins and promiscuous antigens and peptides of *M. tuberculosis* RDs are useful to identify new candidates important for diagnosis and vaccine applications against TB. The experimental validation showed that several proteins and peptides encoded by genes in *M. tuberculosis*-specific RDs were immunodominant and could be useful for diagnostic applications using antibody and cell-mediated immunity-based assays. Two of these antigens (ESXA and ESXB) have been widely tested for diagnostic applications in vitro using IFN- γ assays and have shown diagnostic promise in countries with a low TB burden. *M. tuberculosis*-specific DTH skin responses are also observed with the selected antigens in animals immunized with *M. tuberculosis* and other mycobacteria. Furthermore, the usefulness of the identified antigens as new vaccine candidates was promising when they were expressed in DNA vaccine vectors. All of these results suggest that in silico analysis for immunodominant peptides and proteins could further be extended to include the complete proteome of *M. tuberculosis* to identify all possible candidates for diagnostic and vaccine applications against TB.

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