

The Future of *Mycobacterium tuberculosis*-Specific Antigens/Peptides in Tuberculin Skin Testing for the Diagnosis of Tuberculosis

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A large proportion (about 80%) of the global tuberculosis (TB) burden is found in industrializing and poor countries of Asia and Africa, and children are the most vulnerable part of the population [1]. In these regions, and among the disadvantaged group of people, it is not easy to obtain blood samples and perform highly sophisticated *in vitro* laboratory tests that may not improve the diagnostic efficacy already established by using tuberculin skin test (TST) [2]. Since TST requires minimum laboratory facilities, skin testing remains an attractive alternative in countries with high endemicity of TB. Furthermore, the TST is commonly performed in these settings, and healthcare professionals in these countries are already experienced in injecting the reagents and reading results. Therefore, availability of improved and specific skin test reagents, to be used in TST, is expected to enhance TB control in developing and high burden countries.

It was first demonstrated by Robert Koch, in 1890, that intradermal injection of tuberculin in tuberculosis patients induced inflammatory responses at the site of injection [3]. The tuberculin of Robert Koch (known as old tuberculin) was prepared by dissolving, in a glycerin-containing solvent, the residue from cultures of *Mycobacterium tuberculosis*, which was heated for several hours at 100°C and concentrated 10-fold by evaporation. A relatively purified preparation of tuberculin, known as the purified protein derivative (PPD), was prepared by Seibert et al. [4] in the 1930s and that is the material currently used for identification of subjects infected with *M. tuberculosis* [5]. However, PPD is a crude mixture of >200 proteins present in the culture filtrate of *in vitro* grown *M. tuberculosis*, and contains all types of molecules (species-specific as well crossreactive with the vaccine strains of BCG and non-tuberculous mycobacteria) released from growing and dying cells [5]. Usually, a standard dose (1 or 5 Tuberculin units of PPD, 0.02 or 0.1 µg of protein dissolved in 0.1 ml phosphate buffered saline) is injected intradermally in the forearm and the results are read 48 to 72 hours later by measuring the diameter of induration due to delayed type hypersensitivity (DTH) skin reaction [5]. The induration represents an inflammatory response due to migration of antigen-reactive immune cells (primarily lymphocytes and monocytes) from blood to the site of injection, and release of proinflammatory cytokines by the infiltrating cells [6]. The TST reaction is measured as diameter of induration in millimeters (mm), and reactions of >5 mm induration are considered positive for high risk groups, >10 mm for groups with intermediate risk and >15 mm for groups at low risk of infection with *M. tuberculosis* [5]. The people previously exposed to *M. tuberculosis* are expected to mount a positive DTH skin response to the antigens present in PPD, due to pre-existing cellular immune response, at the injection site [5]. However, the results of TST must be interpreted carefully, because a positive tuberculin test cannot distinguish between active disease, latent infection with *M. tuberculosis*, BCG vaccination, or cross-sensitization by environmental mycobacterial species [7-9]. Inaccuracy of the tuberculin skin test often reflects a low diagnostic specificity due to the presence in PPD of antigens shared by other mycobacterial species [7-9]. Thus, a TB-specific skin test requires the development of new tuberculin(s) consisting of antigens specific for *M. tuberculosis*.

The studies conducted by us have identified a number of immunologically reactive and *M. tuberculosis*-specific protein antigens encoded by genes present in regions of difference (RD), which are absent or deleted in BCG [10-16]. Initial studies with RD-encoded antigens as DTH-inducing reagents were performed in guinea pigs, an animal model that has been extensively used in tuberculosis to study pathogenesis, development of vaccines and identification of diagnostic antigens [17]. The first study in this direction was carried out by Haslov et al. in 1995 using antigens purified from culture filtrate proteins of *M. tuberculosis* [18]. Haslov et al. infected the guinea pigs with *M. tuberculosis* or *Mycobacterium bovis* BCG (Danish strain) and determined DTH skin responses to identify MPT64 (Rv1980c), encoded by RD2, as a molecule specific for tuberculous infection [18]. Hence, they concluded that MPT64 is a promising candidate for a specific diagnostic skin test reagent for human tuberculosis [18]. However, MPT64 belongs to the RD2 region, which is deleted in some (e.g. BCG Danish) but not all BCG strains (e.g. BCG Tokyo), and my not be specific for infection with *M. tuberculosis* [19]. In this respect, study of Haga et al. showed that recombinant MPB64 (a homologue of MPT64 present in *M. bovis*) induced positive DTH responses in guinea pigs injected with live *M. tuberculosis* H37Rv or live *M. bovis* BCG Tokyo (a strain that has RD2 region and secretes MPB64) [20]. To identify the exact DTH-inducing epitopes of MPT64, further studies with overlapping synthetic peptides covering the sequence of full-length MPT64 identified a single DTH-inducing epitope consisting of 15 residues between amino acids Gly-173 and Ala-187 (CE15) [21]. A fine epitope mapping using truncated versions of CE15 indicated the epitope was restricted to 13 residues between amino acids Val-174 to Glu-186, and a chemically modified version of CE15 induced DTH response equivalent to PPD [20]. These studies suggested that synthetic peptides could replace full-length proteins for DTH reactivity. Screening of 56 clinical isolates of *M. tuberculosis* from Danish and Tanzanian patients demonstrated the presence of *mpt64* in all of the strains [21]. Based on these results, the authors suggested MPT64 and its DTH-inducing peptide as possible candidates for a skin test reagent specific for diagnosis of human tuberculosis [21,22]. However, since MPT64 is present in some BCG vaccine strains, it will not be an ideal candidate for diagnostic applications throughout the world. Therefore, to have antigens of universal application, it is required that DTH-

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inducing antigens from those *M. tuberculosis* RDs that are absent in all BCG strains are identified.

Among antigens encoded by genes of RDs deleted/absent in all vaccine strains of BCG, ESAT-6 and CFP10 encoded by RD1 induce *M. tuberculosis*-specific DTH responses in guinea pigs, as shown by positive responses in animals infected with live *M. tuberculosis* by the aerosol and intravenous routes or injected with killed *M. tuberculosis* but negative responses in animals sensitized with live *M. bovis* BCG, live *M. avium* or other non-tuberculous mycobacteria [23-27]. However, all groups of animals showed positive DTH responses to PPD, which demonstrated the non-specificity of PPD [23-27]. Furthermore, a combination of antigens (ESAT-6 and CFP10 or ESAT-6 and MPT64) improved the sensitivity and specificity of the DTH response in cattle [22,23]. These observations support the notion that combination of antigens will improve the sensitivity of RD antigens for in vivo diagnostic application. By using overlapping peptides of ESAT-6, the DTH-inducing epitope of ESAT-6 was mapped to the C-terminus of the protein [22]. Furthermore, as compared to the individual peptides of each protein, testing with a combination of DTH-inducing peptides of ESAT-6 and MPT64 improved the sensitivity of DTH response in *M. tuberculosis*-infected guinea pigs [22]. Thus, suggesting that a combination of DTH-inducing peptides will have better sensitivity in diagnostic application.

The studies with ESAT-6 have been further extended in two most important natural hosts of pathogenic *M. bovis* and *M. tuberculosis*, i.e. cattle and humans, respectively. In *M. bovis*-infected cattle, the sensitivity of DTH response with ESAT-6 was as good as with PPD, with the additional advantage of improved specificity [28]. However, in studies using small number of TB patients, testing with ESAT-6 improved specificity, but reduced sensitivity, as compared to PPD [29]. These results suggest that additional RD antigens/peptides should be identified as new tuberculin for the sensitive and specific diagnosis of TB.

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