Proteins and Peptides Encoded by *M. tuberculosis*-Specific Genomic Regions for Immunological Diagnosis of Tuberculosis

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Tuberculosis (TB) is a major global health problem with about 9 million new cases and 1.7 million deaths annually [1]. The world-wide problem of TB is expected to worsen in the future due to many reasons, including the spread of drug-resistant strains of *Mycobacterium tuberculosis*, and TB-HIV co-infection [1]. In addition to new drugs at affordable prices, the global control of TB requires cost-effective reagents for specific diagnosis and protective vaccines [2]. At present, among the commonly used strategies to control TB in the world are the diagnoses of TB using the purified protein derivative (PPD) of *M. tuberculosis* in delayed-type hypersensitivity (DTH) skin responses, and immunization with *Mycobacterium bovis* BCG as a vaccine. However, the current data demonstrates that both of these modalities have failed to control the world-wide problem of TB [1]. The major limitation of PPD is its inability to differentiate between individuals vaccinated with BCG, infected with *M. tuberculosis* and exposed to environmental non-tuberculous mycobacteria [3]. This is because PPD is a crude mixture of hundreds of proteins present in the culture supernatant of in vitro grown *M. tuberculosis* and contains both species-specific as well as crossreactive antigens [3]. On the other hand, BCG vaccination imparts inconsistent protection against TB in different parts of the world, in people of various age groups and against different clinical manifestations of the disease, which suggest that BCG may be lacking in some important antigens of *M. tuberculosis* required for optimal induction of protective immunity [3]. Therefore, for diagnostic as well as vaccine applications, the identification of *M. tuberculosis*-specific antigenic proteins may be essential [4].

The availability of the complete genome sequence of *M. tuberculosis* in 1998 opened up the door for comprehensive genome comparisons between mycobacterial species to identify species-specific genomic regions, and lead to the identification of 11 *M. tuberculosis* genomic regions deleted/absent in all BCG strains used for vaccination against TB in different parts of the world [5]. It was suggested that these regions could be useful for both specific diagnosis and vaccine applications [6-8]. The analyses of DNA sequence present in these regions of differences (RDs) for the presence of genes and encoded proteins, by using computer-based programs for prediction of open reading frames, have suggested that RD genomic regions deleted/absent in all BCG strains could encode 89 *M. tuberculosis*-specific proteins [9]. As given below, studies have been performed to characterize these proteins for diagnostic applications, using sera and peripheral blood mononuclear cells from TB patients and healthy subjects in antibody and cell mediated immunity assays.

To obtain the RD proteins for immunological investigations, attempts were made first to obtain them as recombinant proteins [9]. These procedures have required the in vitro amplification of RD genes using polymerase chain reaction, cloning of the amplified genes in plasmid vectors to generate recombinant plasmids, expression of the cloned genes in *Escherichia coli* after transforming them with the recombinant plasmids, and purification of recombinant RD proteins from the recombinant *E. coli* cell lysates (free of the *E. coli* components) to homogeneity [10-12]. However, the results of these experiments have shown that problems were encountered at each of the above steps, and therefore, all of the targeted proteins could not be obtained in purified form and in quantities sufficient for immunological investigations [13,14].

To overcome the problems associated with the recombinant production of RD proteins, an alternative approach has been used by employing synthetic peptide chemistry to obtain overlapping peptides covering the sequence of each RD protein [15]. This strategy was successful in synthesizing peptides covering the sequence of all targeted RD proteins absent in BCG [15]. These peptides overlapped by 10 amino acids with the neighboring peptides thus minimizing the missing of linear peptide epitopes recognized by either T cells or antibodies, which usually recognize peptides of less than 10 aa [16]. Conceptually, the use of synthetic peptides in cell mediated immunity assays has no problems because T cells (the effector/indicator cells in cell mediate immunity assays) recognize only linear epitopes, whereas it may be problematic for epitopes recognized by antibodies, because antibodies are usually considered to recognize conformational epitopes. However, recent studies have shown that antibodies may also recognize linear epitopes [17]. Therefore, in our studies, we have used synthetic peptides of RD proteins in both types of assays using 96-well micro titer plates. However, an important difference in using them for antibody and cellular reactivity has been the use of individual peptides and pools of peptides, respectively. The pooling of peptides in case of antibody assays does not work well because of the competition for binding of the peptides to solid support during the coating step of the well in enzyme-linked immunosorbent assays (ELISAs); whereas, pooling of the peptides does not seem to matter much in case of T-cell assays (cellular reactivity) because of the requirement of the binding of peptides to human leukocyte antigens on the surface of antigen presenting cells before the epitope is presented to T cells [16]. This step selects the relevant peptide from the pool and thus minimizes the effect of pooling in T-cell responses.

The evaluation of RD peptides for antibody reactivity in ELISA, with sera from TB patients and healthy subjects, identified three peptides of diagnostic relevance, i.e. aa 346-370 of Rv3876, aa 241-265 of Rv1508c and aa 325-336 of Rv1516c, which had significantly strong antibody reactivity with sera from TB patients than healthy subjects (% positives = 66 to 93%) than sera from healthy subjects (% positives = 10 to 28%).
The diagnostic potential of four antigens encoded by Mycobacterium tuberculosis-specific RD1 region genes (PE35, PPE68, CFP10, ESAT-6) and RD9 region gene Rv3619c was further investigated in cellular assays using DTH responses in guinea pigs [24]. The results showed that mycobacterial sonicates containing species-specific and cross-reactive antigens, induced positive DTH responses in M. tuberculosis, M. bovis BCG, M. avium and M. vaccae injected guinea pigs. However, the purified recombinant proteins PE35, PPE68, CFP10 and ESAT-6 elicited positive DTH responses in M. tuberculosis-injected group but not in BCG, M. avium and M. vaccae-injected guinea pigs, whereas Rv3619c elicited positive DTH 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 responses [24]. Thus, these antigens may be useful in the specific diagnosis of tuberculosis, and therefore could provide a better alternative to the currently used PPD-based skin test in humans.

Acknowledgments

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References