Evaluation of *Mycobacterium tuberculosis* specific RD antigens for delayed type hypersensitivity responses in guinea pig

Mamta Kalra, Gopal Krishen Khuller, Javaid Ahmad Sheikh & Indu Verma*
Department of Biochemistry, Post Graduate Institute of Medical Education and Research, Chandigarh 160012, India

Received 21 April 2009; revised 6 October 2009.

Tuberculin skin test (TST), an age old method is based on measuring delayed-type hypersensitivity (DTH) response to purified protein derivative (PPD). However, inspite of simplicity, ease and cost effectiveness, the usefulness of PPD test is limited due to its inability to distinguish among a protective immune response, latent infection and active tuberculosis disease. On the other hand, a skin test based on RD antigens would add advantages of a high specificity of antigens with the logistics of a skin test. However, except few reports, *in vivo* data of intradermal use of RD antigens for skin testing is limited. Therefore, in the present study, four *M. tuberculosis* (Mtb) specific antigens (ESAT6, CFP10, CFP21 and MPT64) were evaluated for their diagnostic utility based on DTH response. These antigens alone and their multiple combinations induced strong DTH response in Mtb infected guinea pigs and the response was negligible in BCG vaccinated and sham immunized animals.

**Keywords**: Diagnosis, DTH response, PPD, RD antigens, Tuberculosis

Deciphering of the complete genome sequence of *M. tuberculosis* and comparative analysis with other mycobacterial species have led to the identification of several putative vaccine candidates and diagnostic agents. Protein antigens encoded by genomic regions of *M. tuberculosis* designated as Regions of Difference (RD) that are absent from most environmental species along with the vaccine strain BCG have been identified. Restriction of these antigens to *M. tuberculosis* complex makes them attractive for species specific immunodiagnosis.

Genome-wide comparison between *M. tuberculosis* and *M. bovis* reveals that of all RD sequences only RD1, RD2 and RD3 are present in *M. bovis* and RD4-RD16 are missing even in the parent strain from which BCG has been derived. Hence, RD1-3 appears to be of special importance. Of these deletions too, RD3 has been demonstrated to be present in only few clinical isolates, therefore much attention has been focused towards RD1 and RD2 sequences. Early secreted antigenic target-6 (ESAT6) and culture filtrate protein-10 (CFP10) have been identified as immunodominant antigens encoded by RD1. On the other hand, culture filtrate protein-21 (CFP21) and *M. tuberculosis* protein (MPT64) from RD2 have been shown to be potent antigens on the basis of immunoreactivity studies in various models.

An age old diagnostic assay based on Mycobacterium specific activation of cell mediated arm of host immunity is tuberculin skin test (TST). The test is based on measuring delayed-type hypersensitivity (DTH) responses to the intradermal injection of purified protein derivative (PPD). However, the usefulness of PPD is limited by its lack of sensitivity and specificity for infection with *M. tuberculosis*. In addition, TST is unable to clearly distinguish among a protective immune response, latent infection and active disease. However, in spite of all odds, PPD based skin test is still used worldwide to detect *M. tuberculosis* infection due to its simplicity, ease, cost effectiveness and applications in the underserved areas. In view of these advantages, the use of specific RD antigens instead of PPD may promote the diagnostic significance of skin test. In the present study, ESAT6, CFP10, CFP21 and MPT64 RD antigens induced strong DTH reactions in infected guinea pigs, but erythematic response to these antigens was negligible in BCG vaccinated and sham immunized animals.

**Materials and Methods**

**Animals** — Dunkin Hartley outbred (n=38) female guinea pigs weighing 300-400g each, were procured...
from Agriculture University, Hisar (India). All animal experiments were carried out in accordance with the rules and regulations set forth by the Institute Animal Ethics Committee. Animals were housed in cages kept in negative pressure regulated animal isolators and were fed on standard pellet diet (Hindustan Lever Ltd., Mumbai) and water ad libitum.

**Bacterial cultures — Mycobacterium tuberculosis** H$_{37}$Rv and *M. bovis* BCG originally obtained from National Collection of Type Cultures (NCTC), London were used in the study and maintained on Lowenstein Jensen’s (LJ) medium in the laboratory.

**Purification of *M.tuberculosis* complex specific proteins —** Culture filtrate proteins were isolated by growing *M. tuberculosis* H$_{37}$Rv in liquid synthetic Youman’s medium as a stationary pellicle culture at 37°C for 4-5 weeks. Filter (0.22μm) sterilized cell free culture filtrate obtained was concentrated and Mtb specific antigens were identified by the resolution of proteins on SDS-PAGE (16%), followed by blotting with monoclonal/monospecific antibodies (MoAbs) available against ESAT6 (HYB76-8), CFP10 (K8494), CFP21 (K8493) and MPT64 (L24B4). These four proteins were purified from the culture filtrate of *M. tuberculosis* H$_{37}$Rv using anion exchange chromatography followed by preparative SDS-PAGE and electroelution as described earlier$^{14}$.

Briefly, 200mg of concentrated culture filtrate was loaded on to DEAE sepharose CL-6B column followed by elution of bound proteins in a step gradient fashion using increasing concentrations of NaCl (0-300mM). To locate Mtb specific antigens among different elution gradients, peak fraction of each gradient was subjected to ELISA with antibodies against these proteins. Protein (5-10 mg) of the chromatography gradient containing desired *M. tuberculosis* specific protein was resolved by preparative SDS-PAGE using gel percentage of 13 to 18% depending on the molecular weight of the protein to be purified. After completion of the run, fine horizontal strips (≤1mm wide) wereexcised from the desired part of the gel and individual strips were subjected to electroelution at 200V for 4 h. Each eluted sample was then tested for the presence of purified protein on analytical SDS-PAGE followed by silver staining.

**Characterization and identification of purified proteins —** Purity and apparent molecular mass of the purified proteins was determined by SDS-PAGE analysis. However, absolute identity was established by reactivity with MoAbs/PAbs in ELISA. Each of the elute showing single band on acrylamide gel in the range of 6, 10, 21 and 24kDa was probed with respective specific antibody in indirect ELISA. N-terminal sequencing was also carried out for MPT64. In addition, electrospray mass spectrometric characterization of all the four proteins was done at Dr. J.T. Belisle’s lab, Colorado State University, Colorado, USA. Finally, purified proteins were quantified by micro BCA method and stored at -20°C till further use.

**Delayed type hypersensitivity responses in guinea pigs —** Female outbred guinea pigs of Dunkin Hartley strain were divided into three groups of four animals each. One group was infected intramuscularly (im) with 10$^5$ CFUs of *M. tuberculosis* H$_{37}$Rv suspended in 0.1ml of sterile phosphate buffered saline (PBS; pH 7.2). Another group received intradermal injections of 0.1ml PBS containing 2×10$^5$ CFUs of vaccine strain *M. bovis* BCG. These animals were referred to as BCG vaccinated. The third group was left untreated as control naive group. Skin tests were performed 28 days later with 10 tuberculin units (10 TU) of PPD (1TU = 0.02 μg; RT23) and 1μg of each of the native ESAT6, CFP10, CFP21 and MPT64 individually and the combination of all four.

Skin test responses (diameter of erythema) were measured. A total of 2μg of each antigenic combination was injected intradermally. PPD (10TU) and PBS served as positive and negative controls, respectively. The responses were read 24 h post injection and expressed as diameter of erythema in millimeters.

**Results**

The presence of the four proteins of interest (ESAT6, CFP10, CFP21 and MPT64) was ensured by western immunoblotting of the culture filtrate using monoclonal/polyclonal antibodies available against...
these antigens. The western immunoblotting resulted in the appearance of strong bands in the region of 6, 10, 21 and 24kDa when culture filtrate was probed with specific antibodies (Fig. 1).

Anion exchange chromatography of the culture filtrate following step gradient elution using increasing concentrations of NaCl resulted in efficient separation of proteins in different gradients\(^{14}\). The peak fractions obtained with each gradient were subjected to ELISA using antibodies against each protein to detect the presence of protein(s) of interest in a particular gradient. The results of ELISA indicated the presence of ESAT6 and CFP10 in NaCl (150mM) gradient, whereas MPT64 and CFP21 were eluted in 100 and 50mM gradients, respectively (Fig. 2). Once located in a particular gradient each of the four proteins were then purified by electroelution. The purity and apparent molecular weights of the proteins as determined by SDS-PAGE analysis revealed the presence of single bands in the region 6, 10, 21 and 24kDa with respect to the molecular weight marker. However, absolute identity of the purified proteins was established by reactivity with MoAb/PAb in ELISA. The bands giving maximum reactivity with the respective MoAbs and negligible absorbance with other antibodies of the proteins in the same molecular weight region as well as \(M. \text{bovis}\) BCG antisera were taken to be purified RD antigens. N-terminal sequencing and MS-MS further confirmed the identity of the isolated proteins. Dialyzed and concentrated proteins were finally used for the experiment.

**Delayed type hypersensitivity responses induced by complex specific RD antigens in infected guinea pigs** — Induction of delayed type hypersensitivity reaction based T-cell responses by individual \(M. \text{tuberculosis}\) complex specific antigens and their combination was monitored in guinea pigs injected (im) with virulent \(M. \text{tuberculosis}\). Sham immunized and BCG vaccinated guinea pigs were used as control groups. Four weeks post injection/immunization, DTH response was measured in terms of diameter of erythema developed 24 h after intradermal injection of purified ESAT6, CFP10, CFP21, MPT64, combination of all four antigens and PPD on the shaved backs of guinea pigs. All the individual RD antigens and their combination showed significant induction of DTH response in infected guinea pigs as compared to BCG and sham immunized animals (Fig. 3) indicating induction of \(M. \text{tuberculosis}\) specific DTH responses. On the other hand, PPD was able to mount erythematic responses both in Mtb infected as well as BCG immunized animals due to the presence of cross reactive antigens shared by all mycobacteria. The mean diameter observed in response to the combination was higher than the individual antigens, but the difference was not

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**Fig. 1** — Western immunoblotting of culture filtrate of \(M. \text{tuberculosis}\) H\textsubscript{3}Rv with MoAb/PAbs against RD antigens. [Lane 1- Molecular weight marker, Lane 2- HYB76-8 (\(\alpha\)-ESAT6), Lane 3- K8493 (\(\alpha\)-CFP10), Lane 4-K8483 (\(\alpha\)-CFP21), Lane 5- L24b4 (\(\alpha\)-MPT64)]

**Fig. 2** — Reactivity of peak fractions (NaCl elution gradients) with MoAb/PAbs against ESAT6 (HYB 76-8), CFP10 (K8493), CFP21 (K8483) and MPT64 (L24b4).
statistically significant (Table 1). Moreover, even the combination of all four RD antigens was not recognized by all infected animals.

Considering the importance of protein-protein interactions and immunological interference among various components of multiantigenic cocktails,

Table 1 — Erythematic responses in terms of mean diameter (mm) and number of animals recognized in infected versus immunized groups. 5 mm was taken as the positive cut off for the diameter of erythema.

<table>
<thead>
<tr>
<th>Erythema (diam mm) [Mean ± SD]</th>
<th>No. of animals recognized / total no. of animals</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mtb</td>
</tr>
<tr>
<td>ESAT6</td>
<td>5.25±3.09</td>
</tr>
<tr>
<td>CFP10</td>
<td>5.12±1.54</td>
</tr>
<tr>
<td>CFP21</td>
<td>4.75±1.70</td>
</tr>
<tr>
<td>MPT64</td>
<td>5.5±1.63</td>
</tr>
<tr>
<td>Combination</td>
<td>7.0±2.38</td>
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<tr>
<td>PPD</td>
<td>11±6.05</td>
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differential DTH responses were further investigated using various possible antigenic combinations of these proteins in comparison with PPD. No non-specific reaction was observed in both the groups as negligible response was visible against PBS injections. However, substantial erythematic reactions were observed against PPD in Mtb injected as well as BCG immunized groups due to its non-specific nature. In fact, mean diameter of erythema was found to be comparable in the two groups (7.37mm in BCG group versus 7.73 mm in Mtb injected group). Of note, all the antigenic combinations of RD proteins were able to mount significant DTH responses in Mtb injected animals that were statistically much higher than that observed in BCG immunized animals \( (p<0.0001) \). However, among all combinations evaluated for DTH based skin testing, mean diameter response of the combination of two antigens, ESAT6 + MPT64 was found to be the highest. This combination was found to be recognized by all Mtb injected guinea pigs. Most importantly, erythema induced against ESAT6 + MPT64 (16.5mm) was significantly higher than the reaction observed for even the combination of all four RD antigens (11.0mm) as well as PPD \( (p<0.01) \) highlighting the need of selecting optimal combination of well defined antigens (Fig. 4).

**Discussion**

The classic diagnostic tool for latent tuberculosis infection (LTBI) is the tuberculin skin test (TST), also known as the intradermal Mantoux test since 1910. Its low sensitivity and specificity is the weakest element in its use as a diagnostic aid in the evaluation of cases of suspected active TB. A new generation of immune-based rapid blood tests for diagnosis of TB seems to be a significant upgrade of the century-old TST\(^{15,16} \). Measured by the number of recent publications, interferon \( \gamma \) release assay (IGRA) provides a much-wanted alternative to TST. However, in high-endemic and low-resource settings, the use of IGRA is often not feasible due to logistical and financial limitations, such as costs of the assay, lack of laboratory facilities and skilled personnel. For developing countries, a skin test has obvious advantages over *in vitro* tests. Therefore, in spite of all odds, PPD based skin test is still used worldwide to detect *M. tuberculosis* infection due to its simplicity, ease, cost effectiveness and applications in the underserved areas.

Over the past 10 years major efforts have been dedicated to the development of highly purified *M. tuberculosis* recombinant proteins to be used either as a subunit vaccine or as antigens for a more specific diagnostic skin test\(^{17-22} \). Interestingly, the genes encoding some of these antigens (e.g., ESAT6 and CFP10) are located in the RD1 genetic region of the *M. tuberculosis* genome, a region that is deleted from all BCG strains\(^1 \). Therefore, in principle, these antigens are good candidates for diagnostic tools because, by definition, vaccination with BCG does not generate cross-reactions to them. Hence, in the present study four selected *M. tuberculosis* specific RD antigens and their combination were evaluated for their ability to distinguish *M. tuberculosis* infected guinea pigs from BCG vaccinated and sham immunized animals. Although, ESAT6, CFP10, CFP21 as well as MPT64 have been reported to induce significant DTH responses by various researchers, but comparative analysis of their diagnostic potential as skin test reagents is lacking. Earlier, use of intradermal ESAT6 has been demonstrated to reliably identify cattle infected with *Mycobacterium bovis*\(^{18} \). Recombinant ESAT6\(^{19} \) and MTSA-10 (CFP10)\(^{20} \) elicited marked DTH reactions in guinea pigs infected with *M. tuberculosis*, but not in those immunized with BCG or *Mycobacterium avium*. Weldingh *et al.*\(^{20} \) have demonstrated a negative correlation between the size of the skin test response to ESAT6/CFP10, and the time to onset of clinical disease.

In the present study all four RD antigens induced strong DTH reactions in infected guinea pigs, but erythematic response to these antigens was negligible in BCG vaccinated and control groups highlighting the specificity of these antigens (Fig. 3). However, no
remarkable difference was observed in DTH eliciting potential of the four proteins individually. Surprisingly, even the combination of four antigens did not make a significant difference to the individual response in terms of magnitude as well as number of responders (Table 1). On the contrary, combination of only two specific antigens has been demonstrated to improve the sensitivity of detecting TB infection in guinea pigs. Further, it has been proposed that combining more antigens would be able to enhance the sensitivity of the test without jeopardizing the specificity. However, while combining two or more antigens, it is essential to consider their immunocompatibility in terms of recognition by different HLA alleles and competition for antigen processing and presentation. Therefore, in another set of experiment, various possible combinations of ESAT6, CFP10, CFP21 and MPT64 were used for eliciting differential DTH responses in tuberculous guinea pigs over BCG immunized animals. All RD antigens based combinations showed the development of strong erythema in infected guinea pigs, but maximum extent of hypersensitivity response was observed for the combination of ESAT6 and MPT64. Mean diameter produced against ESAT6 + MPT64 was significantly higher than any other combination (Fig. 4). Of note, individual erythema values for this combination were significantly above the cut off. Of note, individual erythema values for this combination were significantly higher than any other combination. Surprisingly, even the combination of four antigens did not make a significant difference to the individual response in terms of differential DTH responses in guinea pigs that were comparable to PPD, however, the same combination failed to elicit erythematous reactions in M. bovis infected cattle. Moreover, inconsistent recognition of MPT64 in different human studies on the basis of DTH responses in spite of exhibiting significantly high erythematous reactions in guinea pigs highlights the relevance of assays conducted directly in human subjects.

References


