Comparative Evaluation of MPT83 (Rv2873) for T Helper-1 Cell Reactivity and Identification of HLA-Promiscuous Peptides in Mycobacterium bovis BCG-Vaccinated Healthy Subjects

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MPT83 (Rv2873), a surface lipoprotein excreted in the culture of Mycobacterium tuberculosis, is immunoreactive in antibody assays in humans and animals and provides protection as a combined DNA vaccine in mice and cattle. This study was undertaken to determine the reactivity of MPT83 in T helper 1 (Th1)-cell assays, i.e., antigen-induced proliferation and gamma interferon (IFN-γ) secretion, using peripheral blood mononuclear cells (PBMCs) obtained from Mycobacterium bovis bacillus Calmette-Guérin (BCG)-vaccinated and/or Mycobacterium tuberculosis-infected healthy subjects. PBMCs were tested with complex mycobacterial antigens and pools of synthetic peptides corresponding to MPT63, MPT83, MPB70, LppX, PPE68, CFP10, and ESAT-6. The results showed that MPT83 is among the strongest Th1 cell antigens of M. tuberculosis, and it was recognized equally strongly by BCG-vaccinated and by BCG-vaccinated and M. tuberculosis-infected healthy subjects. Furthermore, HLA heterogeneity of the responding donors suggested that MPT83 was presented to Th1 cells by several HLA-DR molecules. The analysis of the mature MPT83 sequence (amino acids [aa] 1 to 220) and its 14 overlapping synthetic peptides for binding prediction to HLA class II molecules and actual recognition of the peptides by PBMCs from HLA-DR-typed subjects in antigen-induced proliferation and IFN-γ assays suggested that Th1 cell epitopes were scattered throughout the sequence of MPT83. In addition, the HLA-promiscuous nature of at least three peptides, i.e., P11 (aa 151 to 175), P12 (aa 166 to 190), and P14 (aa 196 to 220), was suggested by HLA-DR binding predictions and recognition by HLA-DR heterogeneous donors in Th1 cell assays. These results support the inclusion of MPT83 in an antigen cocktail to develop a new antituberculosis vaccine.

Tuberculosis (TB) is a major global health problem, and in spite of international efforts to control the disease, TB remains a major challenge to global public health in the 21st century (69). The most recent estimates by the World Health Organization suggest that the worldwide incidence of TB is increasing, with 9.4 million new cases of TB and 1.7 million deaths in 2009 (69). A combination of better diagnostics, drugs, and vaccines is expected to dramatically alter the TB epidemic (28, 35), but it is suggested that vaccines will have the greatest impact in reducing the incidence of disease (35). Mycobacterium bovis bacillus Calmette-Guérin (BCG), the currently used vaccine in humans to protect against TB, has been in use since 1921. However, it has several disadvantages, including the lack of consistent protection in different parts of the world (31), and may cause TB in immunocompromised subjects, including AIDS patients, who are usually at a very high risk of developing TB (15). Thus, there is an urgent need to identify new and safe vaccine candidates to achieve the global control of TB.

The culture filtrate of Mycobacterium tuberculosis (MT-CF) contains secreted and excreted proteins of M. tuberculosis (37), and immunization with MT-CF protects mice and guinea pigs against challenge with virulent M. tuberculosis (14, 17). Furthermore, the protective potentials of several MT-CF proteins, e.g., Ag85 complex, ESAT-6, CFP10, MPT63, MPT64, MPT83, and MPB70, etc., have been demonstrated in animal models of TB (20, 33, 39, 58, 64, 70, 71). All of these antigens, except MPT83, have also been shown to be immunodominant in humans for recognition by Th1 helper 1 (Th1) cells in an HLA-promiscuous manner and induce the release of Th1 cytokines that correlate with protective immunity (40, 41, 42, 59, 65).

Although MPT83 has been studied previously for antibody responses in animals and humans (16, 30, 32) and found to be protective as a combined DNA vaccine in mice and cattle (20, 64), the information on Th1 cell reactivity of this protein in humans, which is considered the hallmark of protective immunity in TB (3, 7, 8, 18, 19, 61), is nonexistent, particularly in M. tuberculosis-infected healthy subjects. In this study, the Th1 cell reactivity of MPT83 was studied in such a group of subjects living in Kuwait by using peripheral blood mononuclear cells (PBMCs) and a pool of overlapping synthetic peptides covering the sequence of the full-length MPT83 protein. In addition, for comparative evaluation, PBMCs were also tested with complex mycobacterial antigens and several major antigens of M. tuberculosis, i.e., ESAT-6 (Rv3875, ESXA), CFP10 (Rv3874, ESXB), PPE68 (Rv3873), MPB70 (Rv2875), LppX (Rv2945c), and MPT63 (Rv1926c). Furthermore, the permissive and promiscuous nature of MPT83 and its peptides was determined by predicting their binding to several alleles of frequently expressed HLA-DR molecules using computational programs (24, 56, 62, 64).
In addition, the non-HLA-restricted presentation of MPT83 to T cells was determined by studying the actual recognition of the peptides by PBMCs obtained from HLA-DR heterogeneous subjects in Th1 cell assays.

**MATERIALS AND METHODS**

Complex mycobacterial antigens and synthetic peptides. The complex mycobacterial antigens used in this study were irradiated whole-cell *M. tuberculosis* H37Ra and *M. bovis* BCG (29, 50), *M. tuberculosis* culture filtrate (MT-CF) enriched for secreted antigens, and purified *M. tuberculosis* cell walls (MT-CW) (48). MT-CF and MT-CW were kindly provided by J. T. Belisle (Colorado State University, Fort Collins, CO). Fourteen synthetic peptides (25-mer overlapping neighboring peptides by 10 residues) spanning the sequence of full-length MPT83 (Fig. 1) were purchased from Thermo Hybaid GmbH, Ulm, Germany. These peptides, as well as the synthetic peptides covering the sequences of MPT63 (43), MBP70 (10), LppX (5), PPE68 (48), ESAT-6 (48), and CFP10 (53), were synthesized using fluorenlymethoxy carbonyl (Fmoc) chemistry, as described previously (11, 49). The stock concentrations (5 mg/ml) of the peptides were prepared in normal saline (0.9%) by vigorous pipetting, and the working concentrations were prepared by further dilution in tissue culture medium RPMI-1640, as previously described (54).

Study subjects and isolation of PBMCs. The study subjects were *M. bovis* BCG-vaccinated healthy adults randomly selected from the group of blood donors at the Central Blood Bank, Kuwait. The primary immunization with BCG was performed at 4 1/2 years of age, followed by a booster immunization with BCG in PPD skin test-negative subjects. At the time of blood collection, all the donors were PPD skin test positive (>15 mm in diameter) from triplicate wells of negative-control cultures lacking antigen. The responses were considered strong with a percentage of positive responses from 40 to 60% and weak with a percentage of positive responses <40% (43, 48). The statistical analysis was performed using a Z test to identify significant differences (P < 0.05) with respect to the percentage of positive results in response to various antigens in antigen-induced proliferation and IFN-γ secretion assays.

HLA typing of PBMCs. PBMCs were HLA typed genomically by using sequence-specific primers in PCR, as described previously (47). In brief, an HLA-DR "low resolution" kit containing the primers to type for DRB1, DRB3, DRB4, and DRB5 alleles was purchased from Dynal AS (Oslo, Norway) and used in a PCR as specified by the manufacturer. DNA amplifications were carried out in a Gene Amp PCR system 2400 (Perkin-Elmer, Cetus), and the amplified products were analyzed by agarose gel electrophoresis, using standard procedures. Serologically defined HLA-DR specificities were determined from the genotypes by following the guidelines provided by Dynal AS.
TABLE 1. Antigen-induced proliferation and IFN-γ results with PBMCs from CFP10/ESAT6 responder and nonresponder healthy subjects in response to complex and single mycobacterial antigens

| Antigen | CFP10/ESAT6 responders | CFP10/ESAT6 non-responders |
|---------|------------------------|---------------------------|
|         | Proliferation δ | IFN-γ γ | Proliferation | IFN-γ |
| M. tuberculosis | 36/38 (95) | 21/23 (91) | 46/49 (94) | 30/31 (97) |
| MT-CF | 36/39 (92) | 23/23 (100) | 49/49 (100) | 30/31 (97) |
| MT-CW | 21/21 (100) | 16/16 (100) | 47/49 (96) | 27/29 (93) |
| BCG | 8/8 (100) | 10/11 (91) | 35/40 (88) | 21/25 (84) |
| MPT63 | 16/39 (41) | 8/23 (35) | 17/49 (35) | 6/28 (21) |
| MPT83 | 27/39 (69) | 17/23 (74) | 28/49 (57) | 12/28 (43) |
| MPB70 | 25/39 (64) | 16/23 (70) | 28/49 (57) | 12/28 (43) |
| LppX | 23/36 (64) | 16/23 (70) | 11/45 (24) | 9/28 (32) |
| MPT83 | 35/39 (90) | 20/23 (87) | 0/48 (0.0) | 0/28 (0.0) |
| CFP10 | 31/39 (80) | 20/23 (87) | 0/48 (0.0) | 0/28 (0.0) |

Table 1. Antigen-induced proliferation and IFN-γ results with PBMCs from CFP10/ESAT6 responder and nonresponder healthy subjects in response to complex and single mycobacterial antigens.

δ A positive response was defined as antigen-induced proliferation with an SI (defined in Materials and Methods) of ≥2. γ A response was considered positive if the IFN-γ concentration in a culture stimulated with antigen minus the IFN-γ concentration in a culture without antigen was ≥1.5 IU/ml.

TABLE 2. ProPred analysis for prediction of HLA-DR binding regions in MPT83 sequence

| HLA-DR allele | Allele binding in predicted regions (aa range) |
|---------------|---------------------------------------------|
|               | 1-220 | 1-12 | 19-27 | 59-68 | 81-89 | 95-104 | 107-119 | 122-130 | 132-140 | 143-166 | 189-204 | 208-217 |
| DRB1.1 | 2/2 | 2/2 | 1/2 | 0/2 | 0/2 | 0/2 | 1/2 | 1/2 | 1/2 | 2/2 | 2/2 |
| DRB1.3 | 7/7 | 2/7 | 0/7 | 0/7 | 7/7 | 0/7 | 5/7 | 0/7 | 0/7 | 7/7 | 6/7 | 7/7 |
| DRB1.4 | 2/9 | 0/9 | 0/9 | 0/9 | 0/9 | 0/9 | 0/9 | 0/9 | 0/9 | 0/9 | 4/9 | 3/9 |
| DRB1.7 | 2/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 2/2 |
| DRB1.8 | 6/6 | 5/6 | 1/6 | 4/6 | 0/6 | 0/6 | 4/6 | 1/6 | 0/6 | 6/6 | 1/6 | 6/6 |
| DRB1.9 | 9/9 | 7/9 | 0/9 | 0/9 | 1/9 | 0/9 | 4/9 | 0/9 | 0/9 | 8/9 | 8/9 | 9/9 |
| DRB1.13 | 11/11 | 7/11 | 1/11 | 0/11 | 0/11 | 0/11 | 4/11 | 0/11 | 0/11 | 9/11 | 6/11 | 11/11 |
| DRB1.15 | 3/5 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 2/3 | 3/3 | 1/3 |
| DRB5.1 | 2/2 | 2/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 | 2/2 | 0/2 | 2/2 |
| P/T α | 51/51 | 25/51 | 3/51 | 5/51 | 8/51 | 9/51 | 26/51 | 3/51 | 1/51 | 39/51 | 29/51 | 47/51 |

Table 2. ProPred analysis for prediction of HLA-DR binding regions in MPT83 sequence.

α P/T, no. of HLA-DR alleles predicted to bind/no. of alleles tested.

β No. of alleles predicted to bind/no. of alleles included in ProPred, in predicted HLA-DR binding regions (aa range) in the sequence of MPT83.
secretion assays (Table 5). HLA-DR typing of the subjects MPT83 in antigen-induced proliferation (Table 4) and IFN-γ secretion assays (Table 5). PBMC donor Antigen-induced proliferation (SI) of PBMCs in response to the peptide pool and individual synthetic peptides of MPT83.

| HLA-DR allele | Binding of MPT83 peptide$^b$ |
|---------------|-------------------------------|
|               | P1   | P2   | P3   | P4   | P5   | P6   | P7   | P8   | P9   | P10  | P11  | P12  | P13  | P14  |
| DRB1.1        | 2/2  | 1/2  | 0/2  | 1/2  | 0/2  | 0/2  | 1/2  | 0/2  | 1/2  | 0/2  | 2/2  | 2/2  |
| DRB1.3        | 2/7  | 0/7  | 0/7  | 0/7  | 0/7  | 0/7  | 5/7  | 0/7  | 7/7  | 5/7  | 0/7  | 6/7  | 7/7  |
| DRB1.4        | 0/9  | 0/9  | 0/9  | 0/9  | 0/9  | 0/9  | 7/9  | 0/9  | 7/9  | 0/9  | 3/9  | 2/9  | 1/9  | 9/9  |
| DRB1.7        | 0/2  | 0/2  | 0/2  | 0/2  | 0/2  | 2/2  | 0/2  | 0/2  | 0/2  | 0/2  | 0/2  | 2/2  |
| DRB1.8        | 5/6  | 1/6  | 0/6  | 0/6  | 0/6  | 0/6  | 5/6  | 1/6  | 1/6  | 0/6  | 1/6  | 6/6  |
| DRB1.11       | 7/9  | 0/9  | 0/9  | 0/9  | 0/9  | 0/9  | 0/9  | 0/9  | 7/9  | 5/9  | 0/9  | 8/9  | 9/9  |
| DRB1.15       | 8/11 | 0/11 | 0/11 | 0/11 | 0/11 | 0/11 | 4/11 | 0/11 | 3/11 | 7/11 | 0/11 | 6/11 | 11/11|
| DRB5.1        | 2/2  | 0/2  | 0/2  | 0/2  | 0/2  | 0/2  | 2/2  | 0/2  | 2/2  | 0/2  | 2/2  |
| P/T$^a$       | 26/51| 3/51 | 0/51 | 0/51 | 0/51 | 0/51 | 28/51| 3/51 | 29/51| 47/51|

$^a$ P/T, no. of HLA-DR alleles predicted to bind/no. of alleles tested.

$^b$ No. of alleles predicted to bind/no. of alleles included in ProPred, in synthetic MPT83 peptides.

Experimental evaluation of MPT83 and its peptides for promiscuous presentation to Th1 cells and comparisons with computational prediction methods. To further analyze the promiscuous presentation of MPT83 and its peptides to Th1 cells, PBMCs of 17 subjects responding to the peptide pool of MPT83 were tested for reactivity to individual peptides of MPT83 and its peptides to Th1 cells. To identify the Th1 cell-reactive and promiscuous peptides of MPT83, PBMCs from all the 17 subjects were tested in both assays for reactivity to individual peptides of MPT83, i.e., P1 to P14. The results showed that positive responses were obtained with all the peptides in 2 to 10 donors in antigen-induced proliferation (Table 4) and 1 to 11 donors in IFN-γ secretion assays (Table 5). Furthermore, the heterogeneity of HLA-DR molecules expressed in the donors suggested that all peptides may have been presented to Th1 cells by more than one HLA-DR molecule (Table 4 and 5).

The results of HLA-DR binding prediction by ProPred, NetMHCII 2.2, Petrochemitric, and IEDB Consensus methods further showed that MPT83 protein was capable of binding to molecules expressed from all HLA-DR alleles included in the analysis (see Table S1 in the supplemental material). In addition, a direct comparison between HLA-DR binding predic-
tion methods and Th1 cell responses of PBMCs to various peptides of MPT83 showed that three peptides were strong stimulators of Th1 cell reactivity, i.e., P11, P12, and P14, of which P11 and P14 were predicted to be HLA promiscuous by three of the four methods, whereas only the Petrochimic method predicted the HLA-promiscuous nature of P12 (see Table S1 in the supplemental material).

**DISCUSSION**

In this study, MPT83 was evaluated, using overlapping synthetic peptides, for Th1 cell reactivity in *M. bovis* BCG-vaccinated and PPD-positive healthy subjects residing in Kuwait. Furthermore, HLA-DR binding prediction analysis of MPT83 and its peptides, as well as HLA-DR typing of MPT83 responders, was performed to determine the promiscuous nature of the full-length protein and its peptides. To my knowledge, this is the first study to determine Th1 cell reactivity of MPT83 and its peptides in HLA-DR-typed *M. bovis* BCG-vaccinated healthy humans and their binding prediction to HLA-DR alleles.

The test systems used to determine Th1 cell reactivity were antigen-induced proliferation and IFN-γ secretion assays. Both of these assays require culture of PBMCs separated from a venous blood sample by density gradient centrifugation (39). However, the measurement of IFN-γ concentration in culture supernatants is considered more sensitive and specific than proliferation assays (7). Since antigen-induced proliferation and IFN-γ secretion have been correlated with protective immunity in tuberculosis (22, 25, 26), in order to identify new candidates for safer subunit vaccines, it is important to identify major *M. tuberculosis* antigens and peptides recognized by human Th1 cells in these assays.

To determine the immunological reactivity of *M. tuberculosis* proteins, full-length proteins purified from cultures of *M. tuberculosis* or purified recombinant proteins, expressed in *Escherichia coli*, have been used (39, 40, 41). However, obtaining full-length proteins from cultures of pathogenic *M. tuberculosis* is extremely hazardous and technically demanding (39). On the other hand, the production of purified recombinant mycobacterial proteins has often been notoriously difficult in *E. coli* (1, 2, 12). To overcome the problems associated with the expression and purification of recombinant mycobacterial proteins, pools of overlapping synthetic peptides have been successfully used in the past to replace recombinant or natural *M. tuberculosis* proteins in Th1 cell assays (40, 41, 55). The inclusion of pools of peptides corresponding to other secreted protein antigens of *M. tuberculosis*, which have been previously characterized for Th1 cell reactivity in *M. bovis* BCG-vaccinated healthy subjects, i.e., MPT63 (43), MPB70 (10), and LppX (5), helped to compare the responses induced by MPT83.

Among the antigens tested in this study, Th1 cell responses to ESAT-6 and CFP10 have been correlated with *M. tuberculosis* infection/latent TB in healthy subjects (23, 46, 63, 66). Therefore, to determine if latent infection with *M. tuberculosis* could have an effect on the Th1 cell responses to various antigens used in this study, the donors were divided into ESAT-6/CFP10 responders and nonresponders to indicate *M. tuberculosis* infection and noninfection, respectively (10, 34). PBMCs from both donor groups had strong responses to all complex mycobacterial antigens without significant differences between the two groups (Table 1), which suggested the suitability of the donors to test for reactivity to single antigens. The responses to MPT83, MPB70, and PPE68 were strong in CFP10/ESAT-6 responders and moderate in nonresponders, without significant differences between the two groups (*P > 0.05*). The only antigen that showed significantly weaker positivity (*P < 0.05*) in ESAT-6/CFP10 nonresponders, compared to responders, was LppX. The overall results suggest that infection with *M. tuberculosis* did not affect the level of Th1 cell reactivity to BCG and most antigens of *M. tuberculosis* used in this study. Furthermore, the study confirms strong to moderate
reactivity of MPB70 and PPE68 in *M. tuberculosis*-infected and noninfected BCG-vaccinated healthy subjects, as reported previously (5, 10, 45, 48), and identifies MPT83 as an immunodominant cross-reactive antigen in humans, like Ag85B and MPB70 (10, 50).

Although the gene for PPE68 is present in the *M. tuberculosis*-specific RD1 segment (13, 36), statistically similar responses in the ESAT-6/CFP10 responders and nonresponders could be due to the presence of an immunodominant epitope (aa 124 to 137), which is conserved in several PPE proteins of *M. tuberculosis*, BCG, and environmental mycobacteria (48, 57). Similarly, one of the immunodominant peptides of MPT83, i.e., P14 (aa 196 to 220), shares 84% to 100% sequence identity with BCG, several species of environmental mycobacteria, and the immunodominant epitope of MPT70 (see Table S2 in the supplemental material). Therefore, in addition to BCG vaccination and infection with *M. tuberculosis*, exposure to environmental mycobacteria may also have contributed to strong and moderate responses to MPT83, PPE68, and MPT70 in CFP10/ESAT-6 responders and nonresponders, respectively. In contrast to the present report, Whelan et al. from the United Kingdom have reported weak responses to MPT83 and MPB70 in *M. bovis*-infected cattle (68). This could be due to species differences and/or a low load of exposure to environmental mycobacteria, as demonstrated for nonrecognition of the cross-reactive epitope of PPE68 (aa 124 to 137) in mice (21) and weak recognition of PPE68 in BCG-vaccinated healthy subjects from Denmark (57).

The strong reactivity of MPT83 in Th1 cell assays suggests that it could be useful in vaccine formulations against TB. However, Th1 cells recognize protein antigens in association with highly polymorphic HLA class II molecules and particularly HLA-DR molecules (38, 51, 52). Therefore, among the requirements for any antigen to qualify as a vaccine candidate is its recognition by an HLA-heterogeneous group of donors. To determine if MPT83 would qualify by this requirement, the PBMCs from MPT83-responding donors were typed for HLA-DR molecules using the server http://www.imtech.res.in/raghava/nhlapred/, which predicts binding to 67 MHC class I alleles. The analysis showed that the MPT83 sequence was predicted to bind 47/67 (70%) alleles, suggesting its HLA-promiscuous nature for recognition by CD8+ T cells as well (data not shown). This suggests that CD8+ T cells may also have contributed to the responses observed.

In conclusion, the results presented in this work demonstrate that MPT83 is a major Th1 cell-stimulating protein of *M. tuberculosis*, with the ability to induce positive responses in HLA-heterogeneous donors and ability to bind HLA molecules promiscuously. Furthermore, the Th1 cell epitopes are scattered throughout the protein sequence. These properties of MPT83 make it an interesting candidate, like Ag85B, HSP65, and MPB70 (39), for inclusion in a vaccine cocktail against TB.

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