Immune responses against *Mycobacterium tuberculosis*-specific proteins PE35 and CFP10 in mice immunized with recombinant *Mycobacterium vaccae*

Hanady A. Amoudy, PhD, DIC, Belghies H. Ebrahimi, BSc, MSc, Abu S. Mustafa, PhD, FRCPath.

**ABSTRACT**

Objectives: To clone and express *Mycobacterium tuberculosis* (M. tuberculosis) proteins PE35 and culture filtrate protein (CFP10) in *Mycobacterium vaccae* (M. vaccae), and subsequently, evaluate the humoral and cellular immunity responses against these recombinant constructs in mice.

Methods: The DNA of PE35 and CFP 10 genes were cloned into the shuttle plasmid pDE22, and the recombinant plasmids were electroporated into *M. vaccae*. The recombinant constructs were then tested for expression of PE35 and CFP10 by Western immunoblotting using rabbit anti-sera. Furthermore, splenocytes and sera from groups of 5 mice immunized with recombinant *M. vaccae* (rVaccae) were tested for cellular and humoral responses in proliferation, and antibody assays. Experiments were carried out in the laboratory of the Faculty of Medicine, Kuwait University, Safat, Kuwait between 2009 and 2011.

Results: The results of Western immunoblot suggested the expression of only PE35. However, splenocyte assays showed positive proliferation in response to peptide pools, and 4 and 5 of the 6 overlapping synthetic peptides covering the sequence of PE35 and CFP10. In addition, positive antibody reactivity was detected with PE35 peptide pool and a single peptide, namely, P2.

Conclusion: The expression of PE35 and CFP10 proteins in rVaccae constructs led to the induction of cellular immune responses to multiple epitopes.

Disclosure. Authors have no conflict of interests, and the work was not supported or funded by any drug company. This study was funded by the Research Sector (grants YM15/08 and MI01/03) and the College of Graduate Studies, Kuwait University, Safat, Kuwait.
Tuberculosis (TB) is a global health concern and a major cause of illness and death worldwide, especially in low and middle-income countries with a high incidence of human immunodeficiency virus infection/acquired immunodeficiency syndrome (HIV/AIDS) co-infection.\(^1\) It is estimated that one-third of the world’s population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*), leading to approximately 9.4 million active cases, and 1.7 million deaths each year.\(^2\) The worldwide problem of TB is due to several factors, such as poverty, overcrowding, breakdown of national infrastructures, immigration of people from high prevalence nations to industrialized countries, and the aging of the world population.\(^3\) The situation is also complicated by a worldwide increase in cases caused by multi-drug resistant and extensively-drug resistant *M. tuberculosis* strains.\(^4,5\) The currently used live and attenuated *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) vaccine against tuberculosis faces several problems, which include variations in the protective efficacy, polymorphism in daughter strains, side effects in immuno-compromised patients, and the implication of misdiagnosis in vaccinated persons using tuberculin skin test, and so forth.\(^6-7\) These limitations support the need of improving BCG, or developing alternative vaccines that are ultimately more effective against TB.\(^8\)

One approach to develop alternative vaccines is to clone and express immunodominant *M. tuberculosis*-specific proteins, deleted from BCG in non-pathogenic mycobacterial vehicles.\(^9,10\) Genetic comparisons between *M. tuberculosis* and BCG have revealed several distinct genomic regions of differences (RD), including a 9.5 kb RD1 segment that is conserved in *M. tuberculosis*, but missing in all BCG strains, and most non-tuberculous mycobacteria.\(^11-13\) The RD1 was shown to encode several secreted proteins with important immunological and protection implications, such as culture filtrate protein (CFP)10, and pulmonary embolism (PE) family-related protein PE35.\(^14-17\) Antigen CFP10 elicits humoral and cellular immunity, and induces protection against *M. tuberculosis* infection in animals.\(^18-20\) On the other hand, proteins of the PE family are known to induce protective immune responses during tuberculous infection in natural host species, and to have potentials in practical applications, as immunological markers of infection, and for the development of vaccines against tuberculosis.\(^21,22\)

Compared with cytosolic and cell wall associated proteins, the secreted proteins are considered more efficient in the induction of protective immune responses.\(^23\) Therefore, we selected the shuttle plasmid vector pDE22, which directs the expression of recombinant genes as secreted proteins.\(^24\) The pDE22 vector, originating from pBLUE SCRIPT SKII polylinker and p16RI vector contains the hygromycin resistance gene marker, heat shock protein (Hsp)60 transcription signal, and secretion signal of the *M. tuberculosis* alpha antigen to direct the expression of recombinant genes as secreted proteins.\(^24\) On the other hand, *M. vaccae* was selected as the vehicle for antigen delivery because of several useful properties of this species. First, *M. vaccae* is a non-pathogenic environmental, and relatively fast growing mycobacterium, which makes it suitable for laboratory manipulation and standardization. Second, *M. vaccae* has been used previously as a host for the expression of recombinant mycobacterial proteins, including superoxide dismutase, major extracellular proteins, and 19 kDa lipoprotein of *M. tuberculosis*.\(^25-27\) In addition, *M. vaccae* has been proven to be an appropriate vehicle to deliver peptide epitopes, and to induce antigen-specific immune responses in animal models.\(^25-28\) In this study, for the secretion of expressed proteins, we cloned PE35 and CFP10 genes in the expression vector pDE22, and the recombinant plasmids were used to transform *M. vaccae*. The expression of the cloned genes in recombinant *M. vaccae* (*rVaccae*) was determined by Western immunoblotting, and the induction of cellular and humoral immune responses to the expressed proteins were studied in *rVaccae*-immunized mice using splenocytes and sera in antigen-induced proliferation and antibody assays. Our objective is to clone and express *M. tuberculosis* - proteins PE35 and CFP10 in *M. vaccae*, and subsequently, evaluate the humoral and cellular immunity responses against these recombinant constructs in mice.

**Methods.** Experiments were carried out in the laboratory of the Faculty of Medicine, Kuwait University, Safat, Kuwait between 2009 and 2011.

**Bacterial strains and plasmids.** The *M. vaccae* was obtained from the American Type Culture Collection (ATCC 15483) (Rockville, MD, USA). Bacterial cells were grown in Middlebrook 7H9 medium supplemented with 2% glucose.\(^10,25\) Plasmids were maintained in *rVaccae* by the addition of 50 μg/ml hygromycin (Invitrogen, Carlsbad, CA, USA). The construction of recombinant plasmids rpGES-TH1-PE35 and rpGES-TH1-CFP10, and the expression of recombinant proteins PE35 and CFP10 in *Escherichia coli* (*E. coli*) have been described previously.\(^19\) The recombinant pDE22-PE35 and pDE22-CFP10 vectors were constructed by double digestion of rpGES-TH1-PE35 and rpGES-TH1-CFP10 constructs using BamHI.
and HindIII restriction enzymes, and subcloning of the released fragments into pDE22 shuttle vector predigested with the same restriction enzymes according to standard methods. The recombinant pDE22 plasmids were propagated in E. coli, and identities of the DNA inserts were confirmed by polymerase chain reaction (PCR) amplification of PE35 and CFP10 DNA using gene-specific oligonucleotide primers (given below), and DNA sequencing according to the methods described previously.

**Oligonucleotides primers.** The forward (F) and reverse (R) oligonucleotide primers for the amplification of PE35 and CFP10 genes by PCR were designed based on their nucleotide sequence in the *M. tuberculosis* H37Rv genome. The DNA sequences of F and R primers for PE 35 and CFP 10 genes are shown below:

**PE35 F** 5’-AATCCGG ATCCATGGAAAAATGTACATGATCCG-3’
**PE35 R** 5’-ACGAAGCTTTTCGGCGAAGA CGCCGGCGGGCGCGCTT-3’
**CFP10 F** 5’-AATCGGATACCGTCATGAG CAGAGATGAAACGGATGCC-3’
**CFP10 R** 5’-ACGTAAGCTTTGAAGGCC ATTTGCGAGGACAG-3’

Another set of F (pDE22F: 5’-AGCGTAAGTAGCGGGGTG-3’) and R (pDE22R: 5’-TGGCTTGTCCAAGGGTGAT-3’) primers were designed, which were based on the sequence of pDE22 shuttle vector, and covered its transcriptional and translational signals. These primers were used in DNA sequencing to confirm the presence, and suitability of the inserts for expression in recombinant pDE22-PE35 and pDE22-CFP10 vectors. All the primers were synthesized commercially (Interactiva Biotechnologies GmbH, Ulm, Germany).

**Transformation of *M. vaccae*.** The recombinant plasmids pDE22-PE35 and pDE22-CFP10 were isolated from *E. coli* cultures on a large scale using Maxiprep EndoFree Plasmid Purification Kits (QIAGEN, Valencia, CA, USA) according to manufacturer’s instructions. Transformation of *M. vaccae* was carried out by electroporation using DNA (15-20 μg of each recombinant plasmid) and a Gene Pulser (BIORAD, Hercules, CA, USA) at the following settings: resistance 1000 ohms; capacitance 25 μFD; and voltage 2.5 V. The colonies of transformed cells were isolated using standard procedures, and cultured in 50 ml 7H9 medium for 10 days in the presence of 50 μg/ml hygromycin. The cells were harvested and washed with 0.9% saline, resuspended in 50 μl 1xTE (10 mM Tris-HCl containing 1 mM EDTA, pH 8.0), and boiled at 95°C for 25 minutes. To confirm the transformation of *M. vaccae* with the recombinant plasmids, 5 μl of the boiled samples were used in the PCR in the presence of gene-specific primers, and the products were analyzed by agarose gel electrophoresis, as described previously.

**Mitogen, mycobacterial sonicates, and synthetic peptides.** The mitogen Concanavalin A (Con A) was purchased from Sigma Chemicals, St. Louis, MO, USA. To prepare *M. vaccae* sonicates, single colonies of wild-type and recombinant *M. vaccae* were grown in 50 ml 7H9 Middlebrook medium (Difco, Detroit, MI, USA), with or without hygromycin. Bacterial cells were harvested by centrifugation at 3500 x g for 15 minutes at 4°C, washed twice with 25 ml phosphate-buffered saline (PBS, pH 7.4), and resuspended in one ml sterile PBS. Bacterial cells were sonicated on ice using an XL Ultrasonic Processor (Heat Systems, NY, USA) with a standard, tapered microprobe tip, as described previously. The protein concentrations in the sonicated supernatants were determined by measuring optical density (OD) at 280 nm. A total of 12 overlapping peptides (25-mers overlapping with neighboring peptides by 10 residues) covering the sequence of PE35 (P1 to P6), and CFP10 (P1 to P6) were synthesized commercially by Thermo Electron GmBH (Ulm, Germany) using fluorenylmethoxycarbonyl chemistry, as described previously. The stock concentrations (5 mg/ml) of the peptides were prepared by dissolving peptide-powders in normal saline (0.9%) by vigorous pipetting, and the working concentrations were prepared in tissue culture medium RPMI-1640, as described previously.

**Analysis of recombinant protein expression in rVaccae.** Mycobacterial sonicates were prepared as discussed earlier. In addition, proteins constitutively expressed in the supernatants of growing mycobacterial cultures were precipitated with 80% ammonium sulfate and re-dissolved in PBS (pH 7.4). Furthermore, sonicated *E. coli* preparations containing recombinant fusion proteins glutathione S-transferase (GST)-PE35 and GST-CFP10 were obtained upon expression of these proteins in *E. coli* transformed with the corresponding pGES-TH1 constructs, as described previously. In all cases, proteins in bacterial cell sonicates and supernatants were analyzed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, as described previously. The resolved proteins were stained with Coomassie Brilliant Blue, or transferred to nitrocellulose membranes for Western immunoblotting using rabbit sera containing antigen-specific antibodies according to standard procedures.
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Ethical approval. Mice were immunized and handled according to established Institutional Animal Care and Use Committees approved protocols at Kuwait University, Kuwait.

Immunization of mice with native and recombinant \textit{M. vaccae}. Six groups of 6 to 8-week-old female BALB/c mice (5 mice in each group) were included in the study. The first group of animals was non-immunized, and the second group was immunized with native \textit{M. vaccae}. The animals of group 3 and group 4 were immunized with rVaccae-pDE22-PE35, and group 5 and group 6 with rVaccae-pDE22-CFP10. For all immunizations, the preparations of native \textit{M. vaccae} and rVaccae were suspended in PBS + 0.05% Tween 80 (PBS-Tween), and 100 μl containing 5x10⁴ colony forming units (CFU) were injected intraperitoneally; boosters were given at 2 week intervals. All the mice of group 1 and group 2 were sacrificed 2 weeks after the primary immunization; whereas 2 animals each of the group 3 and group 5 were sacrificed 2 weeks after the first booster. The mice of group 4 and group 6 received 3 boosters, and all of them were sacrificed 2 weeks after the last immunization. Spleens and sera from all sacrificed mice were collected for further experiments, as given below.

Antigen and peptide-induced proliferation of mice splenocytes. Cells from each spleen were teased out and suspended at 5x10⁴/ml in complete tissue culture medium (RPMI-1640 medium supplemented with 20% fetal bovine serum, and 5x10⁻⁵ M β-mercaptoethanol (ME) (Sigma, St. Louis, MO, USA), and were used in splenocytes proliferation assays according to standard procedures. In brief, 2x10⁵ splenocytes were seeded in 96 well plates containing complete tissue culture medium and stimulated with mitogen ConA, \textit{M. vaccae} sonicate, peptides pools, or overlapping individual peptides covering the sequence of each corresponding RD1 protein. The cell cultures were incubated at 37°C in a humidified atmosphere supplemented with 5% CO₂, pulsed on day 3 with one μCi ³H-Thymidine (Amersham Life Science, Amersham, UK), and harvested 18 hours later on filter mats with Skatron harvester (Skatron Instruments AS, Oslo, Norway). The amount of incorporated radioactivity was determined using liquid scintillation β plate counter (Wallac, Oy, Finland). Proliferation of splenocytes was considered positive if the stimulation index (SI) value was greater than 2. The SI was defined as the average cpm in duplicate wells with antigen/average counts per minute (cpm) in duplicate wells without antigen.

Quantitation of T helper cell (Th)1 (interferon [INF]-γ), Th2 (interleukin [IL]-5), and anti-inflammatory (IL-10) cytokines secreted by mice splenocytes. Supernatants (100 μl) were collected from mice splenocytes cell cultures before being pulsed with ³H-thymidine, and kept frozen until assayed for various cytokines. The 3 cytokines INF-γ, IL-5, and IL-10 were quantified by ELISA using commercial kits (Bender MedSystems, San Diego, CA, USA) according to the manufacturers’ instructions. The 3 kits offered minimum detection limits of 5.3 for INF-γ, 5.0 for IL-10, and 3.3 pg/ml for IL-5. The cytokine response to a given antigen or peptide was considered positive when the concentration was greater than 100 pg/ml, and more than 2 times the concentration in control cultures without antigen stimulation.

Enzyme linked immunosorbent assay (ELISA) for antibodies. The ELISA was performed to detect antibodies against the peptides pools and individual peptides of PE35 and CFP10 in the sera of pre- and post-immunized mice according to standard procedures. In brief, wells of 96-well PolySorp plates (Nunc, Rochester, NY, USA) were coated with peptides (10 μg/ml), blocked with the blocking buffer, incubated with the primary antibody (mouse sera at 1:100) followed by secondary antibody (alkaline phosphatase-conjugated anti-rabbit immunoglobulin G), and addition of substrate for color development, as described previously. The color intensity was determined by measuring the OD at 405 nm. The peptide-coated wells in the presence of secondary antibody alone, that is, without adding primary antibody were used as negative controls. The results were expressed as E/C, which is defined as:

$$E/C = \frac{OD \text{ in peptide-coated wells with primary and secondary antibodies}}{OD \text{ in peptide-coated wells with secondary antibody alone}}$$

The values of E/C >2.0 were considered positive.

Results. Construction of recombinant \textit{M. vaccae}. The DNA inserts of PE35 and CFP10 were restriction digested from rpGES-TH1-PE35 and rpGES-TH1-CFP10 and subcloned into pDE22. The identity of inserts in the resulting recombinant plasmids rpDE22-PE35 and rpDE22-CFP10 was confirmed by PCR and DNA sequencing (data not shown). The \textit{M. vaccae} cells were transformed with rpDE22-PE35 and rpDE22-CFP10 using electroporation and the recombinant colonies of \textit{M. vaccae} were selected on hygromycin-containing medium. The presence of appropriate plasmids in rVaccae was confirmed by PCR.
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using gene-specific primers. Results showed that DNA corresponding to the size of PE35 and CFP10 were amplified from the tested colonies of rVacae-pDE22-PE35 (Figure 1A) and rVacae-pDE22-CFP10 (Figure 1B).

Expression of PE35 and CFP10 in recombinant M. vaccae. Rabbit sera containing anti-PE35 and anti-CFP10 were used as probes in Western immunoblots to detect the expression of PE35 and CFP10 proteins in cell sonicates and supernatants of cultured recombinant M. vaccae. In addition, recombinant proteins expressed from the rpGES-TH-1-PE35 and rpGES-TH-1-CFP10 vectors in E. coli were used as positive controls. The results showed that anti-PE35 and anti-CFP10 antibodies reacted with the corresponding recombinant proteins expressed in E. coli (Figure 2, lane G, data shown for PE35), and anti-PE35 antibodies reacted with a product in the supernatant of rVacae-pDE22-PE35 at approximately 20 kDa (Figure 2, lane C), which corresponded to the expected size of recombinant PE35. This band was absent in the sonicates of rVacae-pDE22-PE35, as well as in sonicates and supernatants of native M. vaccae, and M. vaccae transformed with the parent shuttle vector.


Figure 2 - Western immunoblotting to detect the expression of rPE35 protein in rVacae-pDE22-PE35 using protein-specific antibodies: a) proteins from supernatants of native Mycobacterium vaccae (M. vaccae); b) proteins from sonicates of native M. vaccae; c) proteins from supernatants of rVacae-pDE22-PE35 (long arrow shows a product at approximately 20 kDa reacting to anti-PE35 antibodies); d) proteins from sonicates of rVacae-pDE22-PE35; e) proteins from supernatants of rVacae-pDE22; f) proteins from sonicates of rVacae-pDE22; g) proteins from sonicates of Escherichia coli (E. coli)-pGES-TH1-PE35 (positive control), short arrow shows a product at approximately 40 kDa corresponding to fusion protein GST-PE35 in rE. coli sonicate reacting to the same antibodies; M) pre-stained low molecular weight protein marker.
Table 1 - Mitogen, antigen- and peptide-induced proliferation of splenocytes from non-immunized mice, and mice immunized with native Mycobacterium vaccae (M. vaccae), or rVaccae-pDE22-PE35.

<table>
<thead>
<tr>
<th>Splenocytes stimulated with</th>
<th>Number of positive mice</th>
<th>Native M. vaccae (n=5)</th>
<th>rVacce-pDE22-PE35</th>
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<tr>
<td></td>
<td>Non-immunized mice (n=5)</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>Immune mice</td>
<td></td>
</tr>
<tr>
<td>ConA</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>M. vaccae sonicate</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>PE35 peptides pool</td>
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<td>0</td>
</tr>
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<td>PE35 P1</td>
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<td>PE35 P4</td>
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<td>PE35 P5</td>
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<td>PE35 P6</td>
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<td>rVaccae - recombinant M. vaccae, ConA - Concanavalin A, P - peptide</td>
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Table 2 - Mitogen, antigen- and peptide-induced proliferation of splenocytes from non-immunized mice, and mice immunized with native Mycobacterium vaccae (M. vaccae), or rVaccae-pDE22-CFP10.

<table>
<thead>
<tr>
<th>Splenocytes stimulated with</th>
<th>Number of positive mice</th>
<th>Native M. vaccae (n=5)</th>
<th>rVacce-pDE22-CFP10</th>
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<tr>
<td></td>
<td>Non-immunized mice (n=5)</td>
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<td></td>
<td></td>
<td>Immune mice</td>
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<tr>
<td>ConA</td>
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<td>5</td>
<td>3</td>
</tr>
<tr>
<td>M. vaccae sonicate</td>
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<td>0</td>
<td>3</td>
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<tr>
<td>CFP10 peptides pool</td>
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<td>0</td>
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<td>P6</td>
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<tr>
<td>rVaccae - recombinant M. vaccae, ConA - Concanavalin A, CFP - culture filtrate protein, P - peptide</td>
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Table 3 - Antibody reactivity to Mycobacterium vaccae (M. vaccae), sonicate, peptides pool, and individual peptides of PE35 in sera of mice immunized 4 times with rVaccae-pDE22/PE35.

<table>
<thead>
<tr>
<th>Serum from mouse no.</th>
<th>M. vaccae sonicate</th>
<th>PE35 peptides pool</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
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<th>P6</th>
</tr>
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<tbody>
<tr>
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<tr>
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<tr>
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<td>1.3</td>
<td>0.9</td>
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<td>0.7</td>
<td>0.7</td>
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<td>1.0</td>
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rVaccae - recombinant M. vaccae, P - peptide
Humoral immune response in immunized mice against recombinant proteins. To study humoral responses in mice, sera were collected from the non-immunized and immunized mice, and tested for antibodies to *M. vaccae* sonicate, peptides pools and individual peptides of PE35 and CFP10 using ELISA. The results showed that positive antibody responses to *M. vaccae* sonicate were observed only in immunized mice injected with native or rVaccae after primary, as well as booster injections (Table 3, data shown for mice given 3 boosters). However, antibodies to peptides pools and individual peptides of PE35 and CFP10 proteins were not detected in sera from non-immunized mice, or mice immunized with native *M. vaccae* (data not shown). When tested with peptide pools and individual peptides of PE35 and CFP10, sera from 2/5 mice receiving 3 boosters with rVaccae-pDE22-PE35 showed positive antibody responses (E/C >2) to the peptides pool, and the individual peptide P2 of PE35 protein (Table 3), but mice immunized with rVaccae-pDE22-CFP10 did not show positive antibody responses to the peptides pool, or individual peptides of CFP10, even after 3 boosters (data not shown).

Discussion. In this study, we attempted to express 2 *M. tuberculosis*-specific and immunodominant RD1 proteins, that is, PE35 and CFP10 in non-pathogenic *M. vaccae* for immunological evaluation in mice. Both of these proteins are secreted naturally by *M. tuberculosis* during in vitro growth,\(^\text{40,41}\) and are considered major T-cell antigens for the induction of protective immune responses against *M. tuberculosis*.\(^\text{15,42}\) To mimic the natural expression, the coding DNA sequences of PE35 and CFP10 proteins of *M. tuberculosis* were cloned in the shuttle vector pDE22. This vector contains the secretion sequence motif of *M. tuberculosis* alpha antigen, which is expected to drive the secretion of the cloned recombinant proteins to the outside milieu.\(^\text{24}\) Non-pathogenic *M. vaccae* was transformed with recombinant pDE22, and selected on hygromycin-containing medium because pDE22 has hygromycin resistance gene.\(^\text{24}\) In addition to the known adjuvant properties of mycobacteria, *M. vaccae* was chosen as a vehicle for antigen delivery because of many other characteristics that this mycobacterium, particularly, possesses. First, *M. vaccae* is an environmental, non-pathogenic, and relatively fast grower (it grows in 10 days compared with 4 weeks for BCG), which makes it suitable for experimental standardizations and manipulations. Second, *M. vaccae* has the ability to allow stable maintenance and expression of heterologous DNA, which is an essential characteristic of a recombinant host.\(^\text{43,45}\) In addition, *M. vaccae* modulates the immune response against tuberculosis in favor of protective Th1-type,\(^\text{46,47}\) and whole heat-killed *M. vaccae* has shown promise as a clinical immunotherapeutic agent for TB treatment.\(^\text{45,48}\)

The expression of the recombinant proteins in rVaccae constructs was determined by Western immunoblotting using rabbit sera containing antigen-specific antibodies.\(^\text{19}\) Since recombinant proteins in rVaccae were expected to be actively secreted out by the bacterial cells, proteins present in culture supernatants, in addition to cell sonicates were used in Western immunoblots. Furthermore, sonicates of recombinant *E. coli* cells containing recombinant PE35 and CFP10 were used as positive controls.\(^\text{19}\) The results showed that anti-PE35 and anti-CFP10 antibodies reacted with the corresponding recombinant proteins expressed in *E. coli* with a strong signal, suggesting that *E. coli* expressed a large quantity of these proteins. However, the results with rVaccae/pDE22-PE35 suggested a low level of expression of PE35 because anti-PE35 antibodies showed a faint product at approximately 20 kDa in the culture supernatant, but not in the cell sonicate of rVaccae/pDE22-PE35 (Figure 2). However, this reactivity was specific because no antibody reactivity with a protein of the expected size was observed in either supernatants, or sonicates of native *M. vaccae*, or recombinant *M. vaccae* transformed with parent pDE22 (Figure 2). The size of the detected protein in the supernatants of rVaccae/pDE22-PE35 was 20 kDa, which is close to the expected size of the recombinant PE35 expressed from pDE22-PE35, that is, 18 kDa, 10 kDa for PE35,\(^\text{50}\) and 8 kDa for the secretion signal motif from the shuttle vector.\(^\text{24}\) On the other hand, the expression of CFP10 in rVaccae-pDE22-CFP10 could not be confirmed by Western immunoblotting, which could be due to very low level of expression. Apparently, it was not due to any problem with the cloned DNA because DNA sequencing showed that both PE35 and CFP10 DNA inserts had 100% matching with the expected sequence in *M. tuberculosis* genome,\(^\text{50}\) and both the inserts were in the frame with the promoter and intervening sequences.

To evaluate for the induction of antigen-specific immunological responses, BALB/c mice were immunized with rVaccae/pDE22-PE35 and rVaccae/
pDE22-CFP10 constructs. Cellular immune responses were evaluated using splenocytes in proliferation assays, which indicate T helper-1 type response, and correlate with protective immunity.\textsuperscript{14} The results showed that positive proliferation responses to peptide pools covering the sequence of PE35 and CFP10 were observed in mice only after 3 booster immunizations. However, positive responses to \textit{M. vaccae} sonicate and the T-cell mitogen ConA were observed with splenocytes of all mice after the first immunization, and as expected, the splenocytes from non-immunized mice proliferated to ConA only (Table 1 & Table 2). These results suggest that the lack of splenocytes proliferation to the peptides of PE35 and CFP10 in mice receiving less than 3 boosters was not due to technical reasons. Furthermore, positive cellular responses to peptides pools of PE35 and CFP10 in mice receiving 3 boosters with rVaccae, suggests that both PE35 and CFP10 were expressed in rVaccae, but most probably at low levels, which necessitated multiple injections to sensitize sufficient number of cells to lead to a positive response. The low level expression may also be the reason for the fact that Western immunoblotting could not detect the expression of CFP10 in the culture supernatants of in vitro grown rVaccae-pDE22-CFP10.

To confirm the results obtained with the peptides pools and determine the number of epitopes inducing a positive cellular response, splenocytes from non-immunized and immunized mice were tested with individual peptides of PE35 and CFP10. The results showed that non-immunized and mice receiving less than 3 boosters did not respond to any of these peptides. However, splenocytes from mice receiving 3 boosters responded to 4 and 5 of the 6 peptides of PE35 and CFP10. These results confirm that the responses to peptide pools were not experimental artifacts, and several epitopes of these proteins were involved, as has been previously shown in \textit{M. tuberculosis}-infected humans and guinea-pigs and mice immunized with DNA vaccine constructs of these proteins.\textsuperscript{31,44,49-51} Furthermore, bioinformatics analysis, based on binding to HLA-DR molecules, has also predicted the presence of several T-cell epitopes in these proteins.\textsuperscript{30,52} Interestingly, splenocytes from BALB/c mice immunized with a DNA vaccine construct of PE35 (pUMVC6/PE35) also responded to the same peptides of PE35, that is, P1, P3, P4, and P5 [14], as found in this study (Table 1).

In addition to cellular immune responses, humoral immune responses have been recently shown to contribute to protective immunity against TB.\textsuperscript{35-35} The induction of humoral immune responses to recombinant antigens was evaluated by measuring antibodies in ELISA using sera from mice. Like cellular responses, antigen-specific antibodies were detected only in sera of mice that received 3 boosters with rVaccae/pDE22-PE35. However, only 2 of 5 mice in this group contained a positive antibody titer to the peptide pool of PE35, and to its individual peptide P2 (Table 3). On the other hand, the absence of detectable levels of anti-CFP10 antibodies in the sera of mice immunized with rVaccae/pDE22-CFP10 could again be due to very low expression level of protein. Alternatively, the \textit{M. vaccae}-derived protein is subject to post translational modifications, and it is possible that lipid or carbohydrate moieties influence its immune recognition, and thus, the nature of antibodies induced,\textsuperscript{43,44} which may not be recognized by overlapping peptides used in this work.

The expression of TB proteins in recombinant \textit{M. vaccae} is an attractive strategy for vaccine production. However, according to the results of this and other studies,\textsuperscript{44} low level expression of the recombinant proteins imposes an important limitation on the development of this approach. To obtain high-level expression, other expression systems should be tested, such as the system based on the iron-containing superoxide dismutase (SOD) of \textit{M. tuberculosis}, as a carrier protein. The gene encoding SOD can be expressed at high levels in recombinant mycobacterial systems, accounting for as much as 10% of the total cell protein.\textsuperscript{31,44}

In conclusion, this study confirms the in vitro and in vivo expression of PE35, as well as immunogenicity of both PE35 and CFP10 recombinant proteins in \textit{M. vaccae}; however, the expression is low.

Acknowledgment. The authors gratefully acknowledge the supplier of mice from the Animal Resource Centre, Faculty of Medicine, Health Sciences Centre, Kuwait University, Kuwait.

References

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