Besides being the most widely used vaccine directed against tuberculosis (TB) worldwide, *Mycobacterium bovis* BCG is also the most controversial vaccine in current use. Its protective efficacy varies widely in different parts of the world. One approach to improving the current BCG vaccine might be to produce recombinant BCG strains that express major antigens encoded by genes that are present in the *M. tuberculosis*-specific region of difference 1 (RD1), such as *pe35*, *cfp10*, and *esat6*. In this study, *pe35*, *cfp10*, and *esat6* genes were cloned into shuttle plasmid pDE22 to generate the recombinant plasmids PDE22-PE35, PDE22-CFP10, and PDE22-ESAT6, which were electroporated into BCG to generate recombinant BCGs (rBCGs). The cellular immune responses (antigen-induced proliferation and secretion of selected T helper 1 [Th1], Th2, and anti-inflammatory cytokines, i.e., gamma interferon [IFN-γ], interleukin 5 [IL-5], and IL-10, respectively) that are specific to the proteins of cloned genes were studied by using spleen cells from mice immunized with native BCGs and rBCGs and synthetic peptides covering the protein sequence of the cloned genes. The results showed that the spleen cells did not secrete IL-5, whereas IL-10 was secreted in response to peptides of all three proteins from mice immunized with rBCGs only, suggesting expression of the cloned genes and *in vivo* priming of spleen cells to the expressed proteins. However, in Th1 cell assays that correlate with protective cellular immune responses, i.e., antigen-induced proliferation and IFN-γ secretion, only mice immunized with rBCG-PDE22-PE35 yielded positive responses to the peptides of PE35. These results suggest that rBCG-PDE22-PE35 is the only one of the three vaccines used in this work that is worthy of consideration as a new vaccine candidate against TB.

Tuberculosis (TB) is a major infectious disease problem of global concern. Estimates from the World Health Organization (WHO) suggest that about 9.3 million new cases of TB occur annually (1). Furthermore, tuberculosis is the highest single cause of mortality due to bacterial infections, leading to about 1.7 million deaths worldwide per year (1). Vaccination has long been recognized as an optimal strategy for controlling TB (2). The live attenuated vaccine against TB, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), has been given to neonates or young children around the world since the 1950s. It is known to prevent severe manifestations of the disease, such as meningeval and military TB. Furthermore, the BCG vaccine has many advantages, including a long-standing safety profile, the need for only a single inoculum, superb adjuvant activity, low cost, easy production, and convenient storage (3, 4). Despite all of these advantages and its wide use, BCG vaccination remains controversial (3). Its protective efficacy varies widely in different parts of the world and its impact on the global problem of tuberculosis remains unclear (3). One approach to improving the current BCG vaccine is to express major *Mycobacterium tuberculosis*-specific region of difference 1 (RD1) antigens. To identify the individual open reading frames (ORFs) of the RD1-encoding proteins that are potent for Th1-cell reactivity, peptide pools of 12 ORFs present in the RD1 were tested individually with human peripheral blood mononuclear cells (PBMCs). The results showed that PE35, CFP10, and ESAT6 were among the important antigens that stimulated human PBMCs in the protective Th1 cell assays, i.e., antigen-induced proliferation and gamma interferon (IFN-γ) secretion (5, 6). In addition, when tested with PBMCs in a cattle model of TB, PE35, CFP10, and ESAT6 were also found to be moderate-to-major stimulators of Th1 cells that are present in the peripheral blood of *M. bovis*-infected cattle (7). Moreover, the vaccination of animals, such as guinea pigs and mice, with ESAT6 and CFP10 has been shown to provide protection against challenge with *M. tuberculosis* (8). According to these indications, the *pe35*, *cfp10*, and *esat-6* genes were selected in this study to be cloned into shuttle plasmid pDE22 and used to transform BCG in anticipation of improving its immunogenicity relevant to protection against tuberculosis. The shuttle vector pDE22 was developed by O’Gaora et al. in 1997 (9), and it contains a hygromycin-resistant gene marker in addition to a heat shock protein 60 (hsp60) transcription signal and the secretion signal of *M. tuberculosis* alpha antigen to export the expressed proteins into the extracellular milieu (9).

To study the expression and immunogenicity of the cloned genes in recombinant BCGs (rBCGs), mice were immunized with the live recombinants, and spleen cells were used to study the induction of antigen-specific cellular immune responses, i.e., antigen-induced proliferation and secretion of Th1 (IFN-γ), Th2 (IL-5), and anti-inflammatory (IL-10) cytokines in response to the peptides of proteins expressed by cloned genes.
MATERIALS AND METHODS

Bacterial strains and media. *M. tuberculosis* (strain H37Rv) and *M. bovis* BCG were obtained from the American Type Culture Collection (ATCC), Manassas, VA. Mycobacteria were grown on Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI) supplemented with 0.2% glycerol, 0.05% Tween 80, 10 μg/ml amphotericin B (Fungizone; Gibco), and 10% albumin–dextrose–catalase (ADC) (5% albumin, 2% glucose, 0.004% catalase, and 0.85% sodium chloride) (Gibco-BRL). ABC was grown on solid Middlebrook 7H11 medium (Difco Laboratories) supplemented with 10% oleic acid–albumin–dextrose–catalase (OADC) (0.05% oleic acid, 5% albumin, 2% n-glucose, 0.85% sodium chloride, and 0.004% catalase) (Gibco-BRL). Plasmids were maintained in rBCG by the addition of 50 μg/ml hygromycin to the growth medium. Escherichia coli strain DH5α was grown in Luria broth (LB) (Difco) medium according to standard methods and used for the propagation of native and recombinant plasmids.

Mitogen, antigens, and synthetic peptides. The mitogen concanavalin A (ConA) was purchased from Sigma Chemicals, St. Louis, MO. The complex mycobacterial antigens included BCG sonicates and *M. tuberculosis* culture filtrate (MT-CF) enriched for secreted antigens. MT-CF was produced under NIH contract no. HHSN26220040091C/AD, contract no. AI40092, Tuberculosis Vaccine Testing and Research Materials contract, and was kindly provided by J. T. Belisle (Colorado State University, Fort Collins, CO) (10). A total of 18 peptides (25-mer overlapping with neighboring peptides by 10 residues) covering the sequence of PE35, CFP10, and ESAT6 were commercially synthesized by Thermo Electron Corporation, Germany, using fluorenylmethoxycarbonyl chemistry, as described previously (6), and were provided as powders. The powders were dissolved in sterile phosphate-buffered saline (PBS) (pH 7.0) by vigorously vortexing and were frozen at −20°C in aliquots (1 mg/ml) until used. All antigens, mitogens, and peptides were added to a final concentration of 5 μg/ml in cell culture experiments (11).

Cloning of pe35, cfp10, and esat6 genes in the shuttle plasmid pDE22. The genes of *M. tuberculosis* RD1 proteins PE35, CFP10, and ESAT6 were amplified by PCR using the genomic DNA of *M. tuberculosis* H37Rv and gene-specific primers according to standard procedures (12). The amplified DNA was cloned into pGEM-T Easy (Promega Corp., Madison, WI) and subcloned subsequently into the pGES-TH-1 plasmid for expression in *E. coli*, as described previously (13). After confirming the appropriateness of the inserts in pGES-TH-1, they were excised using the restriction enzymes BamHI and HindIII and subcloned into pDE22 shuttle vector, predigested with the same restriction enzymes, to generate recombinant pDE22-PE35, pDE22-CFP10, and pDE22-ESAT6.

Preparation of recombinant BCG. Native BCG cells were transformed with the recombinant plasmids by electroporation using the Gene Pulser II electroporation system (Bio-Rad, CA) by using procedures described previously (14). The electroporated BCG cells were plated on Middlebrook 7H11 Bacto agar (Difco) supplemented with 10% OADC and 50 μg/ml hygromycin. After 3 to 4 weeks of incubation at 37°C, the individual colonies of growing cells were picked up from agar plates and inoculated further in the liquid 7H9 medium supplemented with 10% ADC and hygromycin (50 μg/ml), according to standard procedures (14). PCR, using gene-specific primers, was performed to check the presence of pe35, cfp10, and esat6 genes in the rBCGs, as described previously (15, 16).

DNA sequencing. Forward (F) and reverse (R) oligonucleotide primers were designed to determine the DNA sequence of transcriptional and translational signals around the multiple cloning site (MCS) and the cloned gene in recombinant pDE22 were designed, and they were synthesized commercially by Interactiva Biotechnologies GmbH, Ulm, Germany. The sequences of these primers were as follows: pDE22F, 5′-AGCGTAGTAG CGGGGGTGTT-3′, and pDE22R, 5′-TGGCTGCTCAAGGGTGTAT-3′. DNA plasmids were isolated from rBCG strains and used as a template for PCR, according to standard procedures (17). The amplified DNA plasmids were purified using the High Pure PCR product purification kit (Roche Diagnostics), according to the manufacturer’s protocol. The purified DNA plasmids were sequenced using the BigDye Terminator v1.1 cycle sequencing kit (3130xl genetic analyzer; Applied Biosystems) and the sequencer (3130xl genetic analyzer; Applied Biosystems), as described by the manufacturer. The obtained sequences were compared with reference sequences covering transcriptional and translational signals, as well as the cloned genes, using the Clustal W2 website (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Immunization of mice. Mice were immunized and handled according to established Institutional Animal Care and Use Committee (IACUC)-approved protocols at Kuwait University, Kuwait. Groups of 6- to 8-week-old female BALB/c mice (5 mice in each group) were immunized intraperitoneally with 5 × 10⁶ CFU of viable native BCG and rBCGs in 100 μl PBS plus 0.05% Tween 80 (PBS-Tween). Four weeks later, all mice immunized with native BCG and two mice from each group immunized with rBCGs were euthanized to collect spleen samples. The remaining three mice from each group immunized with rBCGs were boosted with the same administration route, dose, and rBCGs as in the first immunization. The boosted mice were euthanized 4 weeks later to collect spleen cells. The splenocytes from the euthanized mice were isolated and used in antigen-induced proliferation and secretion of Th1 (IFN-γ), Th2 (IL-5), and anti-inflammatory (IL-10) cytokine assays.

Antigen- and peptide-induced proliferation of mouse splenocytes. Antigen- and peptide-induced proliferation of mouse splenocytes was performed according to standard procedures (18–20). In brief, spleen cells (2 × 10⁶ cells/well) suspended in 50 μl of complete tissue culture medium were seeded into 96-well tissue culture plates (Nunc, Roskilde, Denmark). The plates were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The cultures were pulsed for 24 h on day 3 with 1 μCi of [³H]thymidine (Amersham Life Sciences, Little Chalfont, United Kingdom) and harvested on filter mats with a Skatron harvester (Skatron Instruments AS, Oslo, Norway). The radioactivity incorporated was measured by liquid scintillation counting (21, 22) as counts per minute (cpm). Average cpm was calculated from duplicate cultures stimulated with each antigen or peptide. Cellular proliferation results are presented as the stimulation index (SI), which is defined as follows: SI = average cpm in antigen-stimulated cultures/average cpm in cultures without antigen. SI values of ≥2 were considered to be positive proliferative responses against complex mycobacterial antigens and peptides (21–23).

IFN-γ, IL-10, and IL-5 assays. Supernatants (100 μl) were collected from cultures of mouse splenocytes in 96-well plates before they were pulsed with [³H]thymidine. The supernatants were kept frozen at −70°C until assayed for IFN-γ, IL-10, and IL-5 activity. The amount of cytokines in the supernatants was quantified by enzyme-linked immunosorbent assay (ELISA) using kits purchased from Bender MedSystems, Vienna, Austria, as specified by the manufacturer. The minimum detectable concentrations of IFN-γ, IL-10, and IL-5 by using the kits were 5.3, 5.0, and 3.3 pg/ml, respectively. The secretion of a cytokine in response to a given antigen or peptide was considered positive when the concentration in antigen-stimulated cultures was >100 pg/ml and >2 times the concentration in control cultures without antigen (24).

RESULTS

Construction of recombinant BCG. The recombinant shuttle vectors pDE22-PE35, pDE22-CFP10, and pDE22-ESAT6 were introduced into BCG by electroporation. Hygromycin-resistant colonies were grown in 7H9 medium containing the selective antibiotic. The growing colonies were confirmed for being recombinant using primers that were specific for each RD1 gene in PCR. The results showed that DNA of an expected size was amplified from each tested colony (Fig. 1).

Sequencing of recombinant plasmids isolated from rBCG. The recombinant plasmids pDE22-PE35, pDE22-CFP10, and pDE22-ESAT6 were isolated from transformed BCG colonies and sequenced to check whether they were subjected to any change when incorporated in their final host. Recombinant plasmid DNA

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was prepared and amplified using primers flanking the cloning sites of the shuttle vectors. The amplified products included the hsp65 promoter, the alpha antigen secretion signal (alpha-SS), and the DNA insert sequences. The results showed that all of the cloned genes were intact and in frame for expression in pDE22 (Fig. 2, data shown for PDE22-PE35).

**Antigen-induced proliferation of splenocytes isolated from immunized mice.** The spleen cells from all groups of mice showed positive proliferation (SI ≥ 2) in response to ConA, BCG sonicate, and MT-CF (Tables 1, 2, and 3). Furthermore, all of the mice immunized once with native BCGs and rBCGs had negative proliferation responses (SI < 2) to peptide pools of PE35, CFP10, and ESAT6 and their individual peptides (Tables 1, 2, and 3, respectively). Moreover, spleen cells from mice immunized and boosted with recombinant BCG-pDE22-PE35 showed positive responses to the pool of PE35 peptides and the individual peptides P1 and P3 (Table 1).
Spleen cells from mice that were immunized and boosted with rBCG-pDE22-CFP10 showed positive proliferation (SI > 2) in response to the pool of CFP10 peptides and the individual peptides P1, P2, P4, P5, and P6 (Table 2). Furthermore, the splenocytes of mice immunized and boosted with rBCG-pDE22-ESAT6 did not show antigen-induced proliferation in response to the pool of peptides and the individual peptides of ESAT6 (Table 3). The positive proliferation responses in most of the positive animals were weak (SI = 2 to 4).

Antigen-induced secretion of cytokines by splenocytes of immunized mice. IL-5 secretion was detected only in culture supernatants of splenocytes from all groups of mice in response to ConA but to none of the antigens (data not shown). The cytokines IL-10 and IFN-γ were secreted by splenocytes from all groups of animals in response to BCG sonicate, MT-CF (Fig. 3A and B, respectively), and ConA (data not shown). Furthermore, IL-10 was secreted by splenocytes from mice that were immunized and boosted with rBCG-pDE22-PE35, rBCG-pDE22-CFP10, and rBCG-pDE22-ESAT6 in response to the pool of peptides of PE35, CFP10, and ESAT6, respectively (Fig. 3A). However, splenocytes from mice immunized and boosted with rBCG-pDE22-PE35 secreted only IFN-γ in response to the PE35 peptide pool (Fig. 3B) and the individual peptides P1, P3, P4, and P5 (Fig. 4).

DISCUSSION

The focus of this study was to improve BCG by complementation with genes of the RD1 locus, which encode major antigenic proteins of Mycobacterium tuberculosis. Among the regions deleted during the attenuation of M. bovis to BCG by Calmette and Guérin, the RD1 locus is considered the first deletion and therefore is important for the loss of protective immunity (25). This argument is supported by a previous study, which showed that restoration of the RD1 locus improved the protective efficacy of BCG in animal models of tuberculosis (26). However, the RD1 locus as a whole is also suggested to have a role in the pathogenesis of M. tuberculosis, and the BCG strains complemented with RD1 have shown increased persistence in mice and guinea pigs (27). The RD1 locus contains 9.5 kb DNA and is predicted to encode a total of 14 M. tuberculosis-specific proteins (28). To avoid pathogenicity while retaining the immunogenicity of the RD1, it has been argued that BCG should be complemented with genes encoding individual antigenic proteins of the RD1 (26). In previous studies, using overlapping synthetic peptides corresponding to putative proteins encoded by genes predicted in the RD1 region, PE35, CFP10, and ESAT6 were identified as stimulating protective Th1 cells from mice, cattle, and humans (6, 29, 30). Therefore, in this study, to improve BCG, we complemented BCG with the pe35, cfp10, and esat6 genes of the RD1. All of these genes, which code for secreted proteins in native M. tuberculosis, were cloned into the pDE22 shuttle vector, and BCG cells were transformed with recombinant pDE22 shuttle vector in an attempt to direct the expression of these proteins to the extracellular milieu. The presence of each gene in the rBCGs was confirmed by PCR using gene-specific primers (Fig. 1).

To confirm that cloned inserts were in-frame for expression in BCG, the DNA of recombinant plasmids pDE22-PE35, pDE22-CFP10, and pDE22-ESAT6 was isolated from BCG cultures and processed for DNA sequence analyses of the appropriate regions of each plasmid. The results show that all sequences of the cloned

TABLE 1 Antigen/mitogen-induced proliferation of spleen cells from mice immunized with native BCG and recombinant BCG-pDE22-PE35 and rBCG-pDE22-ORF3 (boosted)

| Spleen cell stimulant† | Native BCG | rBCG-pDE22-ORF3 (boosted) |
|------------------------|------------|---------------------------|
| ConA                   | 5/5        | 2/2                       |
| BCG sonicate           | 5/5        | 2/2                       |
| MT-CF                  | 5/5        | 2/2                       |
| PE35 peptide pool      | 0/5        | 0/2                       |
| P1                     | 0/5        | 0/2                       |
| P2                     | 0/5        | 0/2                       |
| P3                     | 0/5        | 0/2                       |
| P4                     | 0/5        | 0/2                       |
| P5                     | 0/5        | 0/2                       |
| P6                     | 0/5        | 0/2                       |

† ConA, concanavalin A; MT-CF, M. tuberculosis culture filtrate.

The proliferation response was considered positive with a stimulation index (SI) of ≥2.

TABLE 2 Antigen/mitogen-induced proliferation of spleen cells from mice immunized with native BCG and recombinant BCG-pDE22-CFP10

| Spleen cell stimulant† | Native BCG | rBCG-pDE22-CFP10 (boosted) |
|------------------------|------------|---------------------------|
| ConA                   | 5/5        | 2/2                       |
| BCG sonicate           | 5/5        | 2/2                       |
| MT-CF                  | 5/5        | 2/2                       |
| CFP10 peptide pool     | 0/5        | 0/2                       |
| P1                     | 0/5        | 0/2                       |
| P2                     | 0/5        | 0/2                       |
| P3                     | 0/5        | 0/2                       |
| P4                     | 0/5        | 0/2                       |
| P5                     | 0/5        | 0/2                       |
| P6                     | 0/5        | 0/2                       |

† ConA, concanavalin A; MT-CF, M. tuberculosis culture filtrate.

The proliferation response was considered positive with a stimulation index (SI) of ≥2.

TABLE 3 Antigen/mitogen-induced proliferation of spleen cells from mice immunized with native BCG and recombinant BCG-pDE22-ESAT6

| Spleen cell stimulant† | Native BCG | rBCG-pDE22-ESAT6 (boosted) |
|------------------------|------------|---------------------------|
| ConA                   | 5/5        | 2/2                       |
| BCG sonicate           | 5/5        | 2/2                       |
| MT-CF                  | 5/5        | 2/2                       |
| ESAT6 peptide pool     | 0/5        | 0/2                       |
| P1                     | 0/5        | 0/2                       |
| P2                     | 0/5        | 0/2                       |
| P3                     | 0/5        | 0/2                       |
| P4                     | 0/5        | 0/2                       |
| P5                     | 0/5        | 0/2                       |
| P6                     | 0/5        | 0/2                       |

† ConA, concanavalin A; MT-CF, M. tuberculosis culture filtrate.

The proliferation response was considered positive with a stimulation index (SI) of ≥2.
genes were complete and in frame for expression in pDE22 (Fig. 2) and thus suitable for further experiments to determine in vivo expression and immunogenicity. The recombinant constructs (rBCG-pDE22-PE35, rBCG-pDE22-CFP10, and rBCG-pDE22-ESAT6) were evaluated for in vivo expression of ORF proteins by immunizing BALB/c mice and performing cellular responses (antigen-induced proliferation and cytokine assays) using splenocytes from immunized mice. The results show that splenocytes from all groups of mice proliferated and produced cytokines in response to the mitogen ConA and various complex antigens, i.e., sonicates of BCG and the culture filtrate of M. tuberculosis. These results suggest that the procedures used to assess antigen-induced proliferation of splenocytes were working. Therefore, the same procedures were used to determine the proliferation of splenocytes in response to overlapping synthetic peptides of the RD1 ORFs. Ideally, full-length proteins of the RD1 ORFs should have been used in the cellular assays. However, obtaining full-length proteins, pools of synthetic peptides corresponding to each RD1 ORF were used in this study. One of the obvious advantages of this approach is the speed with which peptides can be synthesized and standardized to be tested for stimulating cellular reactivity. Furthermore, immunodominant single peptides can be identified and exact T-cell epitopes defined by subsequent testing of the individual peptides of each ORF, as reported previously (23,34–36). Each synthetic peptide was 25 amino acids (aa) in length and overlapped with the neighboring peptides by 10 residues. The reason for the 10-residue overlap was to greatly reduce the probability of missing T-cell epitopes, which are usually 10 aa in length (37).

In general, immune responses and cytokine secretion levels were lower in mice that were immunized only once with native and recombinant BCG constructs compared to those immunized and boosted with similar constructs. Furthermore, splenocytes from mice that were immunized and boosted with the homologous rBCGs failed to secrete detectable concentrations of Th2 cytokine IL-5, but they could induce secretion of detectable levels of the anti-inflammatory cytokine IL-10, which was secreted in response to the peptide pools of PE35, CFP10, and ESAT6, from splenocytes immunized with the corresponding rBCG. These re-
results confirm those of a previous study by Hanif et al. (18) using DNA vaccine constructs of RD1 ORFs (PE35, CFP10, and ESAT6), which showed that IL-5 was not secreted by the splenocytes of immunized mice in response to peptides of the immunizing ORFs, but that study also failed to detect secretion of IL-10. However, the discrepancy between this study and the study of Hanif et al. for IL-10 results might be due to differences in the vehicles used, i.e., live BCG versus DNA.

The results of Th1-cell assays, i.e., antigen-induced proliferation and IFN-γ secretion, with the splenocytes of mice that were immunized and boosted with homologous rBCGs show that splenocytes proliferated in response to the peptide pools of PE35 and CFP10, but IFN-γ was secreted in response to PE35 only. These results suggest that PE35 is a stronger inducer of Th1 cells when delivered in the context of BCG. In line with these results, the RD1 PE35 has also been reported to induce the strongest IFN-γ responses in mice immunized with the DNA vaccine constructs of PE35, CFP10, and ESA16 (18).

Both antigen-induced proliferation and IFN-γ secretion have been correlated with protective immunity against tuberculosis. In particular, Th1 immune responses mediated by IFN-γ are a prerequisite for mounting efficient protection against M. tuberculosis challenge in mice (38). Humans with mutated IFN-γ receptor genes are highly susceptible to infection by atypical mycobacteria (39), and mice with a disrupted IFN-γ gene neither produce reactive nitrogen intermediates nor restrict the growth of tubercle bacilli, although they do develop granulomas (40). Furthermore, the models, in which the host is unable to produce any IFN-γ necessary for the generation of antimicrobial activity, demonstrate that IFN-γ is essential for the containment of mycobacterial infection (41). Since rBCG-PDE22-PE35 induces both antigen-induced proliferation and IFN-γ secretion, it may be a better vaccine candidate against tuberculosis than are the other two rBCGs.

In addition to strong Th1 cell reactivity, a candidate vaccine against tuberculosis should be able to induce Th1 cell responses against multiple and HLA-promiscuous epitopes of the immunizing antigen (42). This requirement is essential to overcome the limitations imposed by the highly polymorphic nature of HLA class II molecules in human populations, which are required for the presentation of antigenic epitopes by antigen-presenting cells to Th1 cells (37). The results in this study, by testing the individual peptides of PE35 using splenocytes from mice that were immunized and boosted with rBCG-PDE22-PE35, demonstrate that five of the six peptides of PE35, except peptide 6, induced antigen-induced proliferation and/or IFN-γ secretion. Thus, multiple Th1 cell epitopes scattered throughout the sequence of this protein are present. Furthermore, the analysis of the PE35 sequence for peptide sequences that are capable of binding to HLA class II molecules, using a computer-based prediction program (ProPred) (43), suggests that the RD1 PE35 has multiple and HLA-promiscuous T-cell epitopes (Table 4). These observations support the notion that rBCG-PDE22-PE35 deserves to be considered a new candidate vaccine against tuberculosis.

The overall results of this study suggest the potential of rBCG-

![FIG 4](image-url)

**TABLE 4** HLA-DR binding prediction by ProPred analysis for the sequences of complete protein and synthetic peptides (P1 to P6) of RD1 PE35

| Peptide sequence (aa) | 1.1 | 1.3 | 1.4 | 1.7 | 1.8 | 1.11 | 1.13 | 1.15 | 5.1 | All alleles |
|-----------------------|-----|-----|-----|-----|-----|------|------|------|------|-------------|
| PE35 (1–99)           | 2/2 | 6/7 | 9/9 | 0/2 | 3/6 | 9/9 | 10/11| 2/3  | 2/2 | 43/51       |
| P1 (1–25)             | 0/2 | 0/7 | 0/9 | 0/2 | 0/6 | 0/9 | 0/11 | 0/3  | 0/2 | 0/51        |
| P2 (16–40)            | 0/2 | 0/7 | 0/9 | 0/2 | 0/6 | 0/9 | 0/11 | 0/3  | 0/2 | 0/51        |
| P3 (31–55)            | 1/2 | 0/7 | 0/9 | 0/2 | 0/6 | 0/9 | 1/11 | 0/3  | 0/2 | 2/51        |
| P4 (46–70)            | 2/2 | 6/7 | 9/9 | 0/2 | 3/6 | 9/9 | 10/11| 2/3  | 2/2 | 43/51       |
| P5 (61–85)            | 0/2 | 0/7 | 0/9 | 0/2 | 0/6 | 1/9 | 0/11 | 0/3  | 0/2 | 1/51        |
| P6 (76–99)            | 0/2 | 0/7 | 0/9 | 0/2 | 0/6 | 0/9 | 0/11 | 0/3  | 0/2 | 0/51        |
PE35 as a candidate vaccine against TB. This should be confirmed by demonstrating the protective ability of rBCG-PE35 in challenge experiments with live *M. tuberculosis* in animal models of TB. However, supportive evidence is available by using other rBCGs that overexpress cross-reactive antigens (antigens common to *M. tuberculosis* and BCG), i.e., the antigen 85 (Ag85) complex and 38-kDa antigen (44, 45), which show stronger protective efficacy than native BCG in experimental animal models of tuberculosis. Among these, rBCG-30 (rBCG overexpressing Ag85B) has been tested in phase I clinical trials in humans to establish its safety (46). rBCG will retain the attributes of BCG as a vaccine, including a long-standing safety profile, the need for only a single inoculum, superb adjuvant activity, low expense, easy production, and convenient storage. Hence, in spite of the controversies surrounding its use, BCG cannot easily be replaced by another vaccine candidate. Therefore, the improvements to BCG in the form of rBCG expressing *M. tuberculosis*-specific antigens remain among the best choices for the rational design of a vaccine for tuberculosis, and the results reported in this work suggest that rBCG-PE35 may be one of them.

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