Humoral Immune Responses in Mice Immunized with Region of Difference DNA Vaccine Constructs of pUMVC6 and pUMVC7

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Abstract

Background: We aimed to study the antigen-specific antibody responses in mice immunized with recombinant DNA vaccines constructs of pUMVC6 and pUMVC7, containing RD1 and RD9 genes of Mycobacterium tuberculosis. Methods: We immunized mice with the parent and recombinant plasmids and sera were collected and tested for antibodies against pure recombinant proteins of RD1 (PE35, PPE68, EsxA, EsxB) and RD9 (EssV), peptide mixtures of each protein and their individual peptides using enzyme-linked immunosorbent assays. The optical density (OD) values were measured at 405 nm. E/C (OD in antigen-coated wells/OD in antigen uncoated wells) were calculated, and the values of E/C>2 were considered positive. Results: RD1 and RD9 antigen-specific antibodies were detected in sera of mice immunized with the recombinant DNA vaccine constructs (E/C >2.0). With respect to peptide mixtures and single peptides, only PE35 and P6 of PE35; PPE68 and P19, P24 of PPE68 showed antibody reactivity with sera of mice immunized with the corresponding recombinant pUMVC6 and/or pUMVC7 DNA vaccine constructs. Conclusions: The results confirm in vivo expression and immunogenicity of all the five RD1 and RD9 genes cloned in both of the DNA vaccine vectors.

Keywords: Antibodies, antigens, enzyme-linked immunosorbent assays, Mycobacterium tuberculosis, recombinant vaccine vectors

INTRODUCTION

Tuberculosis (TB) is an ancient disease known to humans since the period before the Middle Ages, but it remains a major problem in the world even in the 21st century. The most recent estimates from the World Health Organization shows that, on a global scale, 10.4 million people had active TB disease and 1.4 million people died of TB in 2015. Furthermore, latent infection with Mycobacterium tuberculosis is quite common, with estimates of one-third of the world’s population being infected, and about 10% of the latently infected individuals are expected to develop active TB in their lifetime. Approximately 95% of new TB cases and 98% of all deaths occur in resource-poor developing countries.

We need effective control and eradication strategies to control TB worldwide. This can be achieved using cost-effective methods for specific diagnosis and the immunotherapeutic vaccine that can be administered safely. Especially, effective vaccines are considered the best method to control and eradicate infectious diseases, including TB. Currently, the only TB vaccine available for human use is the Bacillus Calmette–Guerin (BCG).

This vaccine provides significant protection against childhood TB and the most severe and disseminated forms of the disease in adults, but BCG has not been effective against the pulmonary TB (the most common manifestation of the disease in adults accounting for 85% of cases of active TB) in trials conducted mainly in the developing countries. It is considered that the development of a new and more effective vaccine against TB would significantly reduce the burden of the disease in the resource-poor countries of Asia and Africa. Therefore, developing a new vaccine against TB is an urgent need. Several strategies are being explored to develop new vaccines, which include the attenuated strains of M. tuberculosis, improved recombinant BCG, subunit and DNA vaccines, etc.

At present, DNA vaccine is being widely explored. The microbial DNA sequences encoding antigenic proteins

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are expressed in plasmids to be used as DNA vaccine vectors.\textsuperscript{[17]} The strategy includes identifying immunogenic proteins, isolating the encoding genes, cloning them into plasmid expression vectors capable of expressing the cloned genes when injected/delivered into animals intramuscularly.\textsuperscript{[8]} Once into animal muscle cells, the recombinant plasmids transcribe the bacterial DNA sequence into the corresponding mRNA, followed by the expression of recombinant proteins, which sensitize the immune system of the host for induction of antigen-specific cellular and humoral immune responses capable of providing protection against the pathogen.\textsuperscript{[9]}

In previous studies, the major antigenic proteins encoded by genes present in \textit{M. tuberculosis}-specific region of difference (RD) 1 and RD9 genomic sequences, i.e. PE35, PPE68, EsxA, EsxB, and EsxV were identified using cell-mediated immunity assays.\textsuperscript{[10-16]} The DNA vaccine constructs based on these antigens were efficient inducers of antigen-specific cellular immune responses in mice.\textsuperscript{[17]} Although, the role of cell-mediated immunity (CMI) in protection against \textit{M. tuberculosis} infection in experimental animals and humans is well established\textsuperscript{[8-21]} evidence about the role of humoral immunity in the protection against and control of \textit{M. tuberculosis} infection is emerging.\textsuperscript{[22-24]} Therefore, in this study, we evaluated the DNA vaccine constructs of RD1 and RD9 genes for the induction of antigen-specific antibody responses in mice.

**Method**

**Bacteria and vectors**

We used pGEM-T Easy (Promega corporation, Madison, WI, USA) in strain DH5αF’ (Gibco-BRL, Paisley, UK); pUMVC6 and pUMVC7 (Aldeveron, USA) in strain BL21 (Novagen, Madison, WI, USA).\textsuperscript{[25,26]} We grew \textit{Escherichia coli} in standard liquid or solid media with proper antibiotics as shown previously.\textsuperscript{[25,26]} We carried out DNA manipulations, restriction digestions, and bacterial transformations according to standard procedures.\textsuperscript{[27,28]}

**Purification of recombinant proteins**

We amplified the genes of \textit{M. tuberculosis} RD1 (PE35, PPE68, EsxA, and EsxB) and RD9 (EsxV) using genomic DNA of \textit{M. tuberculosis} H₃₇Rv and gene-specific primers by polymerase chain reaction (PCR) according to standard procedures.\textsuperscript{[25]} Then, we cloned the amplified DNA into pGEM-T Easy (Promega Corp., Madison WI., USA) and after that subcloned them into pGES-TH-1 plasmid as described previously\textsuperscript{[26,27]} for the expression in \textit{E. coli}. Then using thrombin protease treatment, affinity chromatography glutathione-S-transferase (GST) columns, and Ni-NTA resin we purified recombinant proteins that has been expressed as fusion proteins with GST, free of the fusion partner, to near homogeneity from \textit{E. coli} cells as described previously.\textsuperscript{[28,29]}

**Synthetic peptides**

Overlapping synthetic peptides (25-mers overlapping neighboring peptides by 10 amino acids) covering the sequence of PE35, PPE68, EsxA, EsxB, and EsxV proteins were designed and synthesized, as described previously.\textsuperscript{[11-13]} Basically, we prepared the stock concentrations (5 mg/ml) of the peptides in normal saline (0.9%) by strong pipetting and mixing, then we diluted the stock in phosphate buffered saline (PBS, pH 7.0) to prepare the working concentrations, as described previously.\textsuperscript{[11-13]}

**Preparation of recombinant DNA vaccine vectors of pUMVC6 and pUMVC7**

Vectors pUMVC6 and pUMVC7 (eukaryotic expression vectors) were used for cloning of PE35, PPE68, EsxA, EsxB, and EsxV genes to make DNA vaccine constructs, as described previously.\textsuperscript{[17]} In brief, we amplified the DNA segments corresponding to PE35, PPE68, EsxA, EsxB, and EsxV by PCR using genomic DNA isolated from \textit{M. tuberculosis} according to standard procedures.\textsuperscript{[17]} Then, first we cloned DNA corresponding to each gene into pGEM-T Easy vector, and their identity was confirmed by restriction digestion with EcoRI, as described previously.\textsuperscript{[17]} Then, we did single digestion of recombinant plasmids pGEM-T/PE35, pGEM-T/PPE68, pGEM-T/EsxA, pGEM-T/EsxB, and pGEM-T/EsxV with BamHI (for subcloning into pUMVC6) and double digestion with BamHI and XbaI (for subcloning into pUMVC7) to release the DNA fragment corresponding to PE35, PPE68, EsxA, EsxB, and EsxV genes with BamHI/BamHI and BamHI/XbaI cohesive termini.\textsuperscript{[17]} After that, we subcloned all the genes into plasmid vectors pUMVC6 and pUMVC7 that been predigested with BamHI/BamHI and BamHI/XbaI, respectively. The recombinant plasmids were propagated in \textit{E. coli} cells, as described previously.\textsuperscript{[17]} According to the manufacturer’s instructions, we used the Qiagen Endofree Mega kits (Qiagen, Valencia, CA, USA) to purify the recombinant and parent pUMVC6 and pUMVC7 plasmids in large quantities.

**Immunization of mice with DNA vaccine constructs**

We immunized groups of 6–8 week old female BALB/c mice (5 mice in each group) intramuscularly with three doses of 100 µg of plasmid DNA in 100 µl saline 3 weeks apart. The plasmids included pUMVC6, pUMVC6/PE35, pUMVC6/PPE68, pUMVC6/EsxA, pUMVC6/EsxB, pUMVC6/EsxV, pUMVC7, pUMVC7/PE35, pUMVC7/PPE68, pUMVC7/EsxA, pUMVC7/EsxB, and pUMVC7/EsxV. The mice were euthanized after 3 weeks of the last immunization, to collect blood and isolate sera to detect antigen-specific antibodies using enzyme-linked immunosorbent assays (ELISA).

**Enzyme-linked immunosorbent assays to detect antibodies in mouse sera**

We performed ELISA to detect antigen-specific antibodies in mouse sera were according to the standard procedures.\textsuperscript{[29]} In brief, the pure recombinant proteins, peptide pools of each protein, and individual peptides of PE35, PPE68, EsxA, EsxB, and EsxV were dissolved in phosphate buffered saline (pH = 7.0) at 10 µg/ml and 100 µl of each was used to coat the wells of 96 well Polysorb plates (Nunc, USA)
overnight at 37°C. The wells were blocked with 100 µl of
blocking buffer (0.17 M boric acid, 0.12 M NaCl, 0.05%
Tween-20, 1 mM ethylenediaminetetraacetic acid and 0.25%
bovine serum albumin, pH = 8.0) for 1 h at 37°C. After three
times washing with distilled water, 50 µl of diluted sera
(diluted 1:100 in blocking buffer) were added and the plates
were incubated for 1 h at 37°C. The wells were washed three
times with distilled water and further washed with 50 µl of
anti-mouse secondary antibody (IgG, IgM, IgA, and Sigma)
conjugated to alkaline phosphatase (1:1000) for 1 h at 37°C.
The wells were washed three times with distilled water, 50 µl
of the substrate (p-nitrophenyl phosphate) (Sigma) was added
to each well and the plates were incubated for 1 h at 37°C. We
used 25 µl of 3 M NaOH to stop the reaction and we measured
the optical density (OD) at 405 nm using ELISA reader.\[29\]
We used wells that not been coated with antigens but coated with
primary and secondary antibodies as a negative controls. We
expressed the results as E/C, that been defined as:

\[
E/C = \frac{OD \text{ in antigen-coated wells with primary and secondary}}{OD \text{ in antigen uncoated wells with primary and secondary}}
\]

We considered the results positive if he values of E/C >2.\[29\]

Ethical approval
Mice were immunized and handled according to approved
Institutional Animal Care and Use Committee protocols at
Kuwait University.

Results
Antigen-specific antibodies in sera of mice immunized
with parent and recombinant plasmids
Antigens-specific antibodies were not detected in the
sera of mice immunized with parent plasmids pUMVC6
and pUMVC7 (E/C <2.0) \[Figures 1a and b\]. However, antigen-specific antibodies against full-length recombinant
proteins were detected in sera of all groups of mice immunized
with the homologous recombinant DNA vaccine constructs
of pUMVC6 and pUMVC7 (E/C >2.0) \[Figures 2 and 3 for sera from mice immunized with recombinant pUMVC6
and pUMVC7, respectively\]. These results confirmed the in vivo
expression and immunogenicity of all the genes cloned in
both vectors. However, relatively high titers of antibodies
in response to four of the five proteins were detected in sera
from mice immunized with recombinant pUMVC6 than
recombinant pUMVC7 \[Table 1\].

Peptides reactive with antibodies in sera of mice
immunized with DNA vaccine constructs
To determine if the linear epitopes were recognized by
antibodies and to identify the epitopes recognized, the mice
sera were further tested with peptide mixtures and single
peptides of each protein in ELISA. The peptide mixture of
PPE68 yielded positive ELISA reactivity with sera from mice
immunized with both vaccine constructs, i.e. pUMVC6-PPE68
and pUMVC7-PPE68 \[Figures 4 and 5b\]. However, the
immunoreactive peptides were variable, i.e., P24 and P19
in mice immunized with pUMVC6-PPE68 \[Figure 4b\] and

Table 1: Average antibody titer with the immunizing antigen

| Vaccine construct | PE35 | PPE68 | EsxA | EsxB | EsxV |
|-------------------|------|-------|------|------|------|
| pUMVC6            | 4.88 | 9.02  | 3.8  | 4.82 | 9.64 |
| pUMVC7            | 3.66 | 3.54  | 4.5  | 3.5  | 8.16 |

*The results shown are average E/C values for 5 mice. E/C is defined as: E/C=OD in antigen coated wells with mouse sera and secondary
antibody/OD in antigen uncoated wells with mouse sera and secondary
antibody. OD: Optical density

Figure 1: Antigen-specific antibody reactivities in pooled sera from five mice immunized with parent plasmids pUMVC6 \[a\] and pUMVC7 \[b\]. Pooled sera from five mice immunized with the parent plasmids were tested for antibody reactivity with enzyme-linked immunosorbent assays using pure recombinant proteins, mixtures of synthetic peptides and individual peptides of PE35, PPE68, EsxA, EsxB and EsxV. Data are presented as E/C were calculated and values >2 were considered positive, as described in materials and methods. The results are presented as average E/C from five mice in each group

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pUMVC7-PPE68 [Figure 5b], respectively. PE35 peptide P6 showed positive antibody reactivity only in animals immunized with pUMVC6-PE35 [Figure 4a]; whereas animals immunized with other vaccine constructs did not show ELISA positivity with peptide mixtures and single peptides of the three other proteins, i.e. ESXA, ESXB, and ESXV [Figures 5a, 4 and 5c-e].

**DISCUSSION**

There is a need to develop new vaccines for TB as the current BCG vaccine failed to protect humans from TB. DNA vaccines are considered attractive in this regard as they elicit protective immune responses, e.g. Th1 biased CD4+ responses and strong CTL responses.\(^{[31]}\) Previously, EsxA (ESAT-6) protein has been evaluated as a DNA vaccine in mice and the results showed that this vaccine elicits Th1-type immunity.\(^{[32]}\) In another study, Maue et al. have shown that an ESAT-6:CFP10 DNA vaccine administered in cattle induced Th1-cell responses and in conjunction with BCG conferred protection against challenge with virulent *Mycobacterium bovis*.\(^{[33]}\) Furthermore, the protection provided by PE and PPE (Rv1806-1807, Rv3812) proteins against *M. tuberculosis* challenge in mice was reported by Vipond et al.\(^{[34]}\) However, recent studies have suggested that both cellular and humoral responses may be required to provide optimal and efficient protection after immunization with vaccines.\(^{[32,24]}\) Although, the induction of cellular responses to all the major antigens of RD1 and RD9, after immunization with the recombinant DNA vaccine constructs, have been reported previously,\(^{[17,35,36]}\) the antibody responses are reported for the first time in this study.
In this study, recombinant DNA plasmid constructs employing two vectors (pUMCV6 and pUMCV7) were used to immunize groups of mice. Although we observed positive antigen-specific antibody (this study) and CMI responses\(^{[17]}\) in response to all of the antigens after immunization of mice with both recombinant plasmids, but recombinant pUMCV6 induced relatively better responses than recombinant pUMCV7. Both vectors share the same promoter (CMV promoter), but they differ in the peptide that is fused with the foreign protein to be expressed as a recombinant fusion protein. The fusion peptide is human interleukin-2 (hIL\(^{-2}\)) secretory protein in pUMCV6\(^{[17]}\) and it is the tissue plasminogen activator (tPA) signal peptide in pUMCV7\(^{[17]}\). The hIL2 secretory protein acted as a better adjuvant for both cellular and humoral immune responses to the fused mycobacterial proteins more effectively than the tPA signal peptide as seen by the improved responses with recombinant pUMCV6.

To determine if the linear epitopes were involved and to identify the epitopes recognized by antibodies induced by immunization with recombinant DNA constructs in mice, the sera were further tested with mixtures of synthetic peptides corresponding to each protein, followed by testing with each peptide. The results showed that antibody titers to peptide mixtures were lower as compared to full-length proteins, and peptide mixtures of only PE35 and PPE68 showed significant antibody reactivity. Furthermore, the antibody reactivity was limited to only one out of six peptides of PE35 and only two of 24 peptides of PPE68. These results suggest that most of the epitopes of RD1 and RD9 proteins recognized by antibodies present in mouse sera were conformational in nature.

![Figure 3: Antigen-specific antibody reactivities in sera from mice immunized with the DNA vaccine constructs pUMVC7/PE35 (a), pUMVC7/PPE68 (b), pUMVC7/EsxA (c), pUMVC7/EsxB (d) and pUMVC7/EsxV (e). Sera from five individual mice (M1 to M5) immunized with the recombinant pUMVC7 plasmids were tested for antibody reactivity with enzyme-linked immunosorbent assays using pure recombinant proteins and mixtures of synthetic peptides of PE35, PPE68, EsxA, EsxB and EsxV, as described in materials and methods. Data are presented as E/C were calculated and values > 2 were considered positive.](image-url)
In another study using rabbits, immunization with purified recombinant proteins has been shown to induce antibody reactivity to multiple peptides of each protein studied in this work. In the case of rabbits, the antigens were vigorously emulsified in incomplete Freund’s adjuvant, which denatures and fragments the proteins into smaller peptides exposing the linear epitopes for B cell recognition and antibody secretion. Thus, these antibodies will be recognized by both full-length proteins as well as small size linear peptides. Whereas recombinant plasmids were injected intramuscularly in isotonic PBS, the muscle cells take up the plasmids and facilitate the expression of the proteins in vivo. This will help to maintain the conformational epitopes of the expressed proteins for recognition by B cells that will eventually differentiate into plasma cells to secrete antibodies, which will mostly recognize the conformational epitopes in the immunizing proteins.

**Conclusion**

All the RD proteins were expressed in mice immunized with both DNA vaccine constructs and induced antibody responses to the immunizing proteins. Furthermore, experiments with synthetic peptides suggest that most of the epitopes recognized by antibodies were conformational in nature. The induction of both cellular and humoral responses to the antigens delivered through DNA vaccine constructs supports their use as new...
vaccines against TB. However, these constructs should be tested in appropriate animal models for protection against *M. tuberculosis* challenge, before they are tested in human clinical trials.

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Nil.

**Conflicts of interest**
There are no conflicts of interest.

![Image](https://example.com/image.png)

**Figure 5:** Antigen-specific antibody reactivities to peptide mixtures and individual peptides in pooled sera from five mice immunized with the DNA vaccine constructs pUMVC7/PE35 (a), pUMVC7/PPE68 (b), pUMVC7/EsxA (c), pUMVC7/EsxB (d) and pUMVC7/EsxV (e). Pooled sera from five mice immunized with the recombinant pUMVC7 plasmids were tested for antibody reactivity with enzyme-linked immunosorbent assays using mixtures of synthetic peptides and single peptides of PE35, PPE68, EsxA, EsxB and EsxV. Data are presented as E/C were calculated and values >2 were considered positive.

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