

Construction and Immunogenicity Study Of A Newly Construct DNA Vaccine Candidate Against Tuberculosis In Mice

Agustine Nengsih Fauzi ^{1*}, Mustaffa Musa ¹, and Zainul Fadziruddin Zainuddin ²

¹Department of Immunology, School of Medical Sciences, University Science Malaysia
16150 Kubang Kerian, Kelantan, Malaysia.

²School of Health Sciences, University Science Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.

Received 1 August 2008 / Accepted 10 December 2008

Abstract. In this study, a plasmid DNA encoding Mtb8.4, 30kDa (Ag85B) and 32kDa (Ag85A) genes of *M. tuberculosis* was constructed as an alternative vaccine candidate against TB. Using assembly polymerase chain reaction (PCR) method, the synthetic gene, designated as VacIV, was constructed from overlapping oligonucleotides of the desired genes. The VacIV gene was cloned into an expression vector, pVAX1[®] to produce a DNA vaccine candidate namely pVaxVacIV. The immunogenicity of pVaxVacIV was then tested in mice. Mice were immunized intramuscularly with pVaxVacIV. Control mice were immunized with the blank vector (pVAX1[®]). The splenocytes were cultured with purified protein derivatives (PPD), rVacIV protein or Mtb8.4 synthetic peptide for lymphocytes transformation test (LIT) and cytokines assay. Sera were also collected to determine the level of serum IgG subclasses. Our results showed that lymphocytes from mice immunized with the pVaxVacIV secreted significantly higher level of interferon gamma (IFN- γ) but not Interleukin-4 (IL-4) compared to the control mice. Mice immunized with pVaxVacIV also showed high stimulation index and high ratio of IgG2a:IgG1 as compared to control group. These results suggested that pVaxVacIV immunogenic in mice and can be further developed as a potential vaccine candidate for TB.

Keywords: Assembly PCR, DNA vaccine, *M. tuberculosis*

INTRODUCTION

Mycobacterium tuberculosis infects about one-third of the world's population, causing approximately 3 million deaths annually (Dye *et al.*, 1999). The disease is due to a respiratory infection (Lowrie *et al.*, 1997) caused by a bacterium, *Mycobacterium tuberculosis* (Lauzardo and Ashkin, 2000). The recent increase in the incidence of TB, particularly antibiotic-resistant tuberculosis underscores the need for an effective vaccine against this important disease (Jacobs, 1994). The only vaccine currently in use is the live, attenuated strain of *Mycobacterium bovis*, Bacille Calmette-Guérin (BCG), that was derived in the early 1920s (Colditz *et al.*, 1994).

Although vaccination with BCG is widely practiced worldwide, the efficacy of this vaccine varies from 0% to 85% in different populations. The efficacy of BCG vaccination reduces over a period of 10 to 15 years, caused there is no prevention against pulmonary TB in adult population (Sterne *et al.*, 1998; Colditz *et al.*, 1995). As a result, there is an urgent need to develop better or improved TB vaccines as an alternative to BCG. DNA vaccine is an example of non-living candidate vaccines which can be a better approaches in vaccine development (Norazmi and Mustaffa, 2004). DNA vaccine is constructed by inserting the most promising antigens under strong promoters in an expression vector and injected into muscle cells (Kumar *et al.*, 2003; Huygen, 2003). DNA vaccination has been shown to induce protective cellular and humoral responses in animal models of tuberculosis and also has been shown to

give long lasting protection (Kumar *et al.*, 2003). Previously, researchers had constructed a DNA vaccine encoding the genes for *M. tuberculosis* secreted proteins MPT64 (23kDa), Ag85B (30kDa) and ESAT-6 (6 kDa) as candidate antigens. The result showed that the most effective secreted protein was Ag85B followed by ESAT-6 and then MPT64 (Kamath *et al.*, 1999). The protein of the Ag85 complex is a major secretion product of *M. tuberculosis* and *M. bovis* and both Ag85A and Ag85B elicit T-cell responses in TB patients (Wiker and Harboe, 1992; Launois *et al.*, 1994). The Ag85 was tested as a DNA vaccine in animal models and shown to induce high cellular response and confer protection in mice and guinea pig models of TB (Ulmer *et al.*, 1998; Huygen, 1996). Recent studies had shown that one of the immunoreactive antigen against T-cell is 8.4kDa protein. This antigen had showed potential to elicit strong Th-1 T cell responses (Coler *et al.*, 2001; Coler *et al.*, 1998)

Herein, we have constructed a DNA vaccine carrying genes encoding for Mtb8.4, 30kDa (Ag85B) and 32kDa (Ag85A) under a strong eukaryotic promoter in the vector used. In this study we investigated the immune responses elicited by these antigens to indicate that the vaccine constructed could be the potential vaccine candidate against TB.

* Author for correspondence:

Mailing address: Department of Immunology, School of Medical Sciences, University Science Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia. Tel: 609 766 4220; Fax: 609 765 3370; Email: leo_tni@yahoo.co.uk

MATERIALS AND METHODS

Construction of VacIV genes by assembly PCR. Thirty-four overlapping oligonucleotides (≤ 40 -mer each) with human and Salmonella codon bias were assembled by assembly PCR to construct a synthetic gene namely VacIV (Norazmi and Mustafa, 2004; Stemmer *et al.*, 1995). The amino acid sequences for each component of the VacIV synthetic gene are listed in Table 1. The VacIV genes were amplified using forward primer (5'-CCAAGCTTCGCCACCATGGATC-CTGTTGAT-3') contained *Hind*III site, a short recognition sequence KOZAK (Kozak, 1986) and start codon, ATG and reverse primer (5'-CAAAGAATTCTCAGGCCTGGGAAGGATC-3') contained *Eco*RI site and stop codon (Figure 1). This PCR was performed using following conditions; 30 cycles at 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 30 seconds. The synthetic VacIV was then cloned into pCR® 2.1-TOPO® vector (Invitrogen, USA). The recombinant plasmid namely pTOPOVacIV was transformed into *Escherichia coli* TOP10 (Invitrogen, USA) and grown in Luria-Bertani (LB) broth media at 37°C for overnight (Sambrook and Russell, 2001). The plasmid was extracted using Qiaprep® Spin Miniprep Kit (Qiagen, USA).

Construction and preparation of pVaxVacIV as a DNA vaccine. VacIV gene was cloned into pVAX1®, an expression vector (Invitrogen, USA) at *Hind*III and *Eco*RI site. The recombinant plasmid designated as pVaxVacIV was transformed into *E. coli* TOP10 (Invitrogen, USA). The clone was confirmed by DNA sequencing analysis. For immunization, the plasmid DNA was prepared using Endofree Plasmid Maxi kit (Qiagen, USA) according to the recommendation by the manufacturer. The purified plasmid was dissolved in TE buffer (10 mM Tris-Cl, 1 mM EDTA at pH8.0). The plasmid concentration was adjusted to 1 µg/µl using TE buffer and stored at -20°C.

Expression and purification of VacIV protein. For the preparation of recombinant VacIV protein, an expression

Table 1. Amino acid sequences for the component of synthetic VacIV gene.

Peptide/antigen	Sequence
Mtb 8.4 (whole peptide)	DPVDAVINTTCNYGQVVAAL NATDPGAAAQFNASPVAQSY LRNFLAAPPQRAAMAAQLQ AVPGAAQYIGLVESVAGSCN NY
30kD(Ag85B) 101-115	P21 LTSELPQWLSANRAV
30kD(Ag85B) 126-140	P26 SMAGSSAMILAAYHP
30kD(Ag85B) 261-275	P53 THSWEYWGAQLNAMK
32kD (Ag85A) 51-110	WDINTPAFEWYDQSGLSVVM PVGGSFYSFDWYQPACGKA GCQTYKWETFLTSELPGLWQ
32kD (Ag85A) 121-160	AVVGLSMAASSALTLAIYHP QQFVYAGAMSGLLDPSQAMG

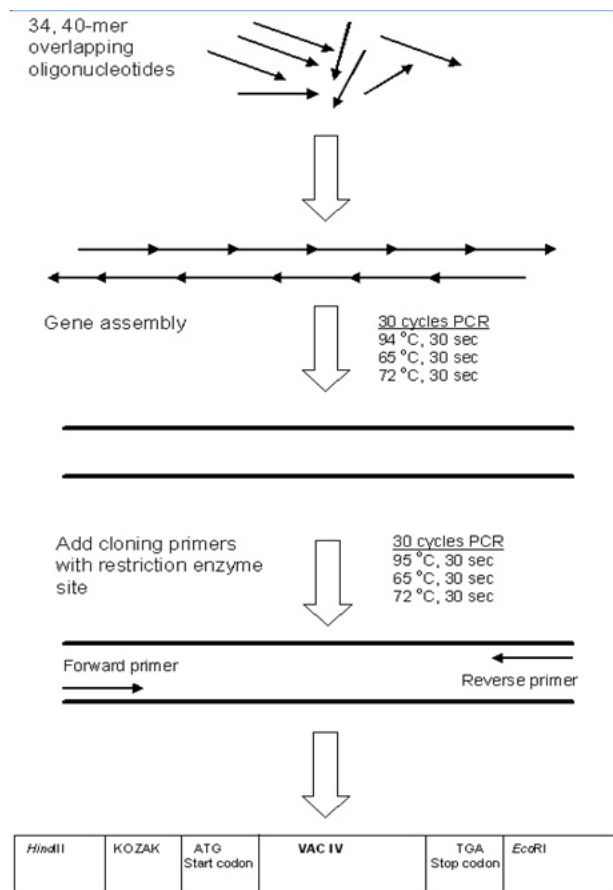


Figure 1. The assembly PCR procedure used to construct a synthetic VacIV gene

vector pPROEX™ HTb was used (Invitrogen, USA). The recombinant plasmid, pROVacIV was transformed into *E. coli* DH5-α (Invitrogen, USA) and grown in LB media at 37°C for overnight. The rVacIV protein was expressed in *E. coli* DH5-α and was induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) (Promega, USA). The 6xhis tagged rVacIV proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography under denaturing conditions cell according to the recommendation by Qiaexpress System kit (Qiagen, USA). The rVacIV protein was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a nitrocellulose membrane and visualized using monoclonal anti-polyHistidine antibody produced in mouse (Sigma, USA) as a primary antibody and peroxidase conjugate goat, anti-mouse polyvalent immunoglobulin (Sigma, USA) as a secondary antibody. The recombinant protein was solubilized and purified with NiNTA purification system under denaturing conditions using 8 M urea and dialyzed by urea gradient dialysis.

Immunization of mice. Six to eight weeks old C57BL/6 female mice were obtained from Animal House, USM Health Campus, Kelantan, Malaysia. All the experiments involving

animals were approved by and performed according to Universiti Sains Malaysia Animal Ethics Committee's guidelines. Mice (five per group) were injected with pVaxVacIV DNA vaccine into both quadricepses with 2x50 µg DNA for three times at 2 weeks interval. Mice were also injected with pVAX1[®] vector as a negative control. Mice sera were taken before and after the immunization for each group.

Lymphocytes proliferation assay. Mice were sacrificed 2 weeks after last immunization by cervical dislocation. Splens from each group were pooled together. The splens were homogenized and single cell suspension was prepared by lysing red blood cells with ACK lysis buffer (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 M Na₂EDTA, pH 7.2) and resuspended in complete RPMI 1640 culture medium (Gibco) with 10 % heat inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were adjusted to a final concentration of 2×10⁶ cell/ml. A volume of 200 µl of cell suspension was added to 96-well plates. The cells were stimulated with 200 µl of rVacIV protein, purified protein derivative (PPD) or Mtb8.4 synthetic whole peptide at a final concentration of 10 µg/ml in RPMI 1640 culture medium. For negative control, the cells were stimulated with 200 µl of RPMI 1640 culture medium and treated in the same manner. The plate was incubated at 37°C in humidified atmosphere of 5% CO₂ for 48 h. The MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] solution of 5 mg/ml was added to the culture, followed by the incubation in 5% CO₂ at 37°C for 4 h. Sodium dodecyl sulphate (20%) dissolved in 50% N,N-dimethylformamide was added to the culture, which were incubated for 12 h at 37°C in 5% CO₂. Finally, the optical density (OD) was read and the stimulation index (SI) was calculated using the formula: stimulation index (SI) = experimental OD/negative control OD. Concanavalin A (ConA) was used in this study as a positive control for lymphocytes responsiveness.

Evaluation of cytokines production in vitro. Single splenocytes suspensions from immunized mice were diluted in 10 % heat inactivated fetal bovine serum (FBS) and RPMI 1640 culture medium to 2 × 10⁶ cells/ml. One hundred microlitre aliquots of cell suspension was cultured in a 96 well-plate and stimulated with rVacIV protein, PPD or Mtb8.4 synthetic peptide at a final concentration of 10 µg/ml in RPMI 1640 culture medium. The cells were incubated for 72 h at 37°C in 5 % CO₂. After 72 h incubation the cell culture supernatant were harvested and were screened for the presence of IFN-γ and IL-4 using ELISA detection system, OptEIAM mouse IFN-γ set and OptEIAM mouse IL-4 set (BD Biosciences, San Jose, CA) according to the manufacturer's instruction.

IgG1 an IgG2a enzyme linked immunosorbent assay (ELISA). Sera obtained from the immunized mice in each group were pooled to determine the level of IgG subclasses by ELISA. A 96 well-plate was coated with 100 µl of rVacIV protein (at a concentration of 10 µg/ml in carbonate-bicar-

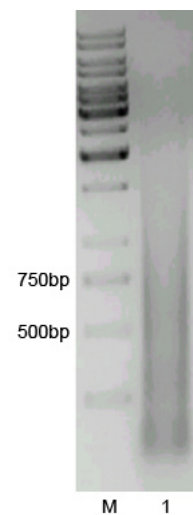


Figure 2. Agarose gel electrophoresis (0.8 % w/v) analysis of assembly PCR product. The assembly PCR was performed by following cycle: 30 cycles at 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 30 seconds. Lane M: 1 KB Ladder, Lane 1: assembly PCR product.

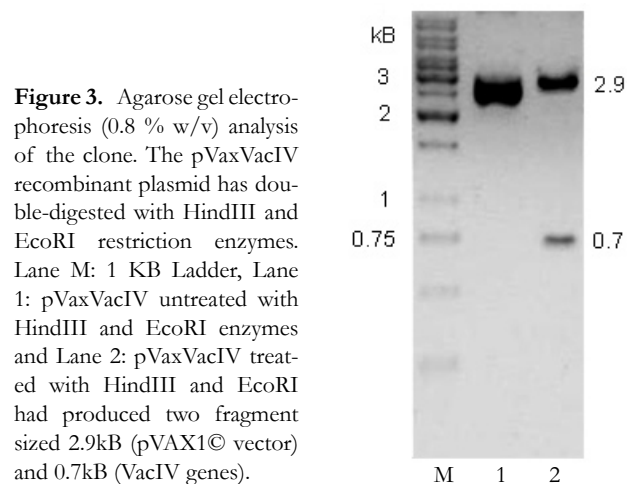


Figure 3. Agarose gel electrophoresis (0.8 % w/v) analysis of the clone. The pVaxVacIV recombinant plasmid has double-digested with HindIII and EcoRI restriction enzymes. Lane M: 1 KB Ladder, Lane 1: pVaxVacIV untreated with HindIII and EcoRI enzymes and Lane 2: pVaxVacIV treated with HindIII and EcoRI had produced two fragment sized 2.9kB (pVAX1© vector) and 0.7kB (VacIV genes).

bonate buffer). The plate was sealed and incubated overnight at 4°C. The wells were then blocked with 200 µl of 1X phosphate saline buffer (PBS) containing 10 % FBS. The plate was again incubated for 1 h at 37°C. One hundred microlitre of mice sera (diluted in 1X PBS at dilution 1:50) was added into the wells and incubated for 2 h at 37°C. The plate then was incubated for 1 h with 100 µl of horse-radish peroxidase conjugate goat-anti mouse IgG1 or IgG2a (diluted with 1X PBS at dilution 1:3000)(Zymed) The reaction was developed by adding tetramethylbenzene (TMB) substrate solution (BD Biosciences, San Jose, CA). The index of antigen specific reactivities was measured according to optical density (OD 450nm). The relative ratio of IgG2a to IgG1 was calculated.

Statistical analysis. The statistical significance of differential finding between experimental groups was determined using the SPSS 11.0 software program for Windows (LEAD Technologies, LTD, UK). Data was considered statistically significant at $p < 0.05$.

Table 2. Analysis of IgG subclasses in mice sera vaccinated with pVAX1© (control group) and pVaxVacIV (test group).

Group	IgG2a	IgG1	IgG2a/IgG1 Ratio
Control	0.777±0.010 ^a	0.010±0.001 ^a	75.32 ^b
Test	0.838±0.015 ^{a*}	0.009±0.003 ^a	89.94 ^{b*}

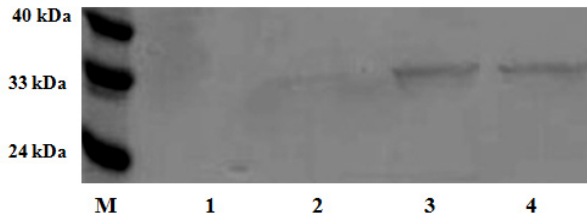


Figure 4. Western blot analysis of the rVacIV-6xHis tagged protein. Lane M: Prestained protein Ladder, Lane 1: pROEX Htb blank vector after 6 hours induction in 1.0mM IPTG (negative control), Lane 2: pROVacIV after 2 hours induction in 1.0mM IPTG, Lane 3: pROVacIV after 4 hours induction in 1.0mM IPTG and Lane 4: pROVacIV after 6 hours induction in 1.0mM IPTG.

RESULTS

Construction of pVaxVacIV DNA Vaccine candidate. The product of assembly PCR was analyzed by electrophoresis using 0.8% agarose gel. The result showed smears around the expected size of the VacIV gene (700 bp) (Figure 2). The VacIV synthetic gene that encoding for Mtb8.4, 30kDa and 32kDa genes was successfully ligated in pVAX1© expression vector to produce a pVaxVacIV DNA candidate vaccine. In order to confirm the correct clones, the recombinant plasmid was screened by restriction enzyme (RE) analysis using two different restriction enzymes: *Hind*III and *Eco*RI. The RE analysis showed that the clone was cut into two fragments with expected size of the VacIV insert (0.7kb) and pVAX1© vector (2.9kb) (Figure 3).

Expression and purification of VacIV protein. The recombinant VacIV protein was expressed and purified in *E. coli* DH5- α after induction with 1 mM IPTG. From the immunoblotting result, the rVacIV protein was successfully expressed with the presence of protein band at expected molecular weight size (30.4 kDa) (Figure 4). In this experiment, blank pROEX™ Htb vector was used as a negative control.

Lymphocytes proliferation response. The immunogenicity of the VacIV gene was studied in mice based on lymphocytes proliferation response and cytokines production in vitro. Several antigens were used which include rVacIV protein (10 μ g/ml), PPD (10 μ g/ml) and Mtb8.4 synthetic whole peptide (10 μ g/ml). The result of the lymphocytes stimulation index (SI) is shown in Figure 5. Splenocytes stimulated with ConA showed an average SI of 26 in tested group (data

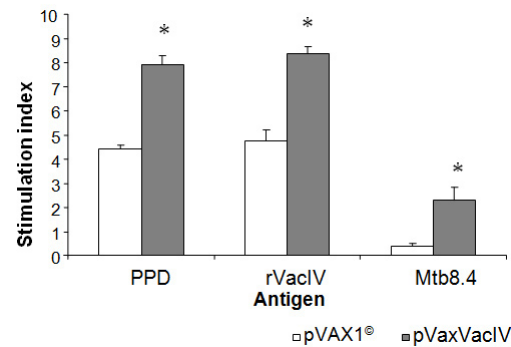


Figure 5. The stimulation index of splenocytes of mice vaccinated with pVAX1© (control group) and pVaxVacIV (test group) with various antigens. Each bar represents the mean of quadruplicate values (mean±standard deviation).

*p<0.05

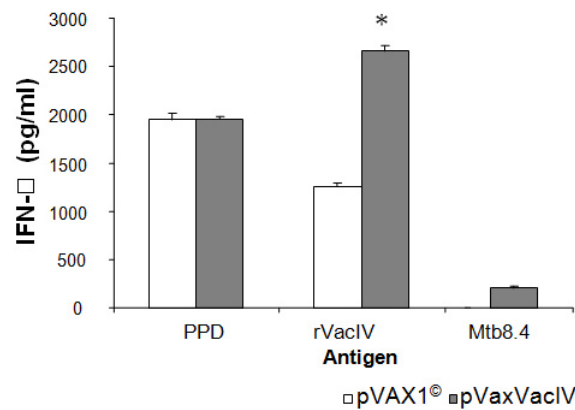


Figure 6. ELISA analysis of IFN- γ of splenocytes in response to PPD, rVacIV protein or Mtb8.4 whole peptide. Each bar represents the mean of quadruplicate values (mean±standard deviation). The experiment was repeated under same condition and the result showed the same pattern of IFN- γ level.

*p<0.05

not shown). Using three different antigens, splenocytes from pVaxVacIV immunized mice showed higher SI compared to splenocytes from the control group (pVAX1© immunized mice)(p<0.05). However, there was no significant difference in SI between rVacIV and PPD but Mtb8.4 antigen alone gave the lowest SI. These results showed that the DNA vaccine constructed was immunogenic in mice based on lymphocytes stimulation.

Cytokines production by splenocytes in vaccinated mice. The IFN- γ production was measured in culture supernatant of splenocytes after re-stimulation with the rVacIV antigen, PPD and Mtb8.4 synthetic peptide. As shown in Figure 6, the highest level of IFN- γ secretion was found in the rVacIV stimulation (2652 pg/ml). This level was significantly higher compared to the control group (1253 pg/ml)(p<0.05). However, there was no significant difference in IFN- γ production after PPD and Mtb8.4 stimulation between the test

and control group. For the IL-4 production, there was low level of this cytokine for all antigen stimulation and there was no significantly different between the test and control group (data not shown). High production of IFN- γ with low IL-4 indicated that the pVaxVacIV mediated Th1 type of immune response.

Production of antibodies in vaccinated mice. As shown in Table 2, the test group ratio of IgG2a:IgG1 was significantly higher than control group which were 89.94 and 75.32 respectively. This result also substantiated the above finding that the humoral response was generated against T-cell mycobacterial multi-epitopes (VacIV) and definitely induced Th1 response.

DISCUSSION

The ability of plasmid DNA to stimulate both exogenous (major histocompatibility complex [MHC] class-II restricted) and endogenous (MHC class-I restricted) antigen presentation pathways as it entering the host had driven to activate both CD4⁺ T cell and CD8⁺ T cell-mediated immune responses (Huygen, 2003). The epitopes-based DNA plasmid encoding CD4⁺ T cell epitopes was used in this study. The T cell epitopes would activate the CD4⁺ and CD8⁺ T cells as a result in response to *M. tuberculosis* infection. The activation of this T cell population would lead to Th1 related cytokines production such as IFN γ and IL-12 (Huygen, 2003; Flynn, 2004). Kamath *et al.* (1999) had demonstrated the use of Ag85 antigen complex (30kDa and 32kDa) in a DNA vaccine showing the effectiveness of this antigen to generate antimycobacterial immunity where the Ag85 complex are more effective as compared to another mycobacterial antigen such as MPT64 and ESAT-6. Coler *et al.* (1998) had discovered that Mtb8.4 is an immunoreactive T cell antigen and it plays an important role in determining outcome of infection with pathogenic mycobacterium.

Hereby, we had demonstrated through our study the immunogenicity of our new DNA vaccine construct based on the induction and generation of immune responses in murine model. The stimulation index of splenocytes proliferation response showed that the T cell lymphocytes proliferated in response to the rVacIV antigen stimulation. This result supported the efficacy of the T-cell epitopes used in this VacIV DNA vaccine construct.

Our findings had shown the increased level of IFN- γ in pVaxVacIV vaccinated mice compared to the control group after re-stimulation with rVacIV antigen. These results have support the facts that this cytokine is involved in inducing a Th1 mediated immune response. The IFN- γ as well as IL-12 cytokines are major costimulatory cytokines in Th1 mediated immune response (Huygen, 2003; Flynn, 2004). Our findings also demonstrated that the Th2-type major cytokines, IL-4 had shown no increase after re-stimulation with rVacIV antigens. This result negates the role of

Th2 mediated immune response. Daoyin *et al.* (2005) had observed the same patterns of IFN- γ and IL-4 cytokines level in mice vaccinated with DNA vaccine carrying the Ag85B antigen, showing that this affect may be associated with a switch to Th1 responses.

In our experiment, we also aimed to observe the effect of IgG2a:IgG1 ratio in response to the VacIV DNA vaccine immunization. This ratio is enhanced in sera of mice vaccinated with this plasmid DNA construct compared to the control group. The higher level of IgG2a subclasses compared to IgG1 indicates the immune response was Th1 type. In murine model, one characteristic effect of the development of the cytokine environment during immune response against any microorganism is the switching between IgG2a and IgG1 antibody isotypes (Mauricio *et al.*, 2005). The production of IgG2a antibodies are part of Th1 type response instead of IFN- γ , IL-12 and IL-2 production. However the actual role of antibodies in immune response to mycobacterium remains uncertain (Flynn, 2004)

The antigens used (rVacIV, PPD and Mtb8.4 pooled peptides) in splenocytes stimulation for both groups of mice (control and test) produced variable values of IFN- γ and IL-4 cytokines and the SI in lymphocytes transformation. From the study, it was observed that the Mtb8.4 pooled peptides did not to induce similar level of lymphocytes proliferation and IFN- γ cytokine production in T-cells compared to the rVacIV protein. This may be due to the rVacIV properties where all the epitopes used in this study were presented in rVacIV protein but not in Mtb8.4 pooled peptides. In addition, the epitopes used in this study were expressed in one single peptide in the vaccine candidate as compared to the Mtb8.4 pooled peptide which consisted of a large number of peptides. The antigen processing of the pooled peptides does not stimulate the primed lymphocytes as well as purified VacIV protein while the use of PPD stimulated the primed lymphocytes. PPD contains the proteins from the epitopes present in VacIV. These results suggested that the primed cells recognize these epitopes in the PPD which is also contained in purified VacIV protein. In this study, we had observed a high background in the control group (samples from pVAX1[®] treated mice) resulted in false-positive results. This might be due to pre-exposure of mice to mycobacterium or cross contamination of samples and reagents during the experiment.

This study had shown the importance of testing the new vaccine candidate in producing the desire effects. It is important to know which type of immune response is mediated by the vaccine immunized because the key in controlling the *M. tuberculosis* infection is cellular immune response, that is activation of CD4⁺ T cell activation and production of major cytokines of Th1 type such as IFN- γ and IgG2a antibodies (Mauricio *et al.*, 2005; Silva *et al.*, 2001).

As a conclusion, vaccination with a newly constructed plasmid DNA (pVaxVacIV) encoding Mtb8.4, 30kDa (Ag85B) and 32kDa (Ag85A) was found to be immunogenic in mice which generated strong Th1 mediated immunity. The data shown in this study provides the evidence that this

vaccine candidate could be one of the potential vaccines in future. Obviously, this promising vaccine candidate has to be tested on another animal model such as guinea pigs to confirm its effectiveness before proceeding to human trials.

ACKNOWLEDGEMENT

We thank the Immunology Department, School of Medical Sciences and School of Health Sciences, University Science of Malaysia for their support and cooperation. This study is funded by Malaysia IRPA grant (No: 305/PPSP/6112208). We also thank to Ministry of Science, Technology and Innovation (MOSTI) for the National Science Fellowship (NSF) awarded.

REFERENCES

- Colditz, G. A., Berkey, C. S., Mosteller, F., Brewer, T. F., Wilson, M. E., Burdick, E. & Fineberg, H. V. 1995. The efficacy of bacillus Calmette-Guerin vaccination of newborns and infants in the prevention of tuberculosis: meta analyses of the published literature. *Pediatrics* 96: 29-35.
- Colditz, G. A., Brewer, T. F., C.S. Berkey, M.E. Wilson, E. Burdick, H.V. Fineberg & Mosteller., F. 1994. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analyses of the published literature. *JAMA*: 271:698-702.
- Coler, R. N., Campos-Neto, A., Owendale, P., Day, F. H., Fling, S. P., Zhu, L., Serbina, N., Flynn, J. L., Reed, S. G. & Alderson, M. R. 2001. Vaccination with the T cell antigen Mtb8.4 protects against challenge with Mycobacterium tuberculosis. *Journal of Immunology* 166: 6227-6235.
- Coler, R. N., Skeiky, Y. A., Vedvick, T., Bement, T., Owendale, P., Campos-Neto, A., Alderson, M. R. & Reed, S. G. 1998. Molecular cloning and immunologic reactivity of a novel low molecular mass antigen of Mycobacterium tuberculosis. *Journal of Immunology* 161: 2356-64.
- Daoyin, Z., Shan, J. & Xudong, L. 2005. Therapeutic effects of Ag85B and MPT64 DNA vaccine in a murine model of Mycobacterium tuberculosis. *Vaccine* 23: 4619-4624.
- Dye, C., Scheele, S., Dolin, P., Pathania, V. & Ravigliione, M. C. 1999. Global burden of tuberculosis: estimated incidence, prevalence and mortality by country. WHO Global Surveillance Monitoring Project. *Journal of American Medical Association* 282: 677-686.
- Flynn, J. L. 2004. Immunology of tuberculosis and its implications in vaccine development. *Tuberculosis* 84: 93-101.
- Huygen, K. 2003. On the use of DNA vaccines for the prophylaxis of mycobacterial diseases. *Infection and Immunity* 71: 1613-21.
- Huygen, K., Content, J. & Denis, O. 1996. Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. *Nature Medicine* 2: 893-8.
- Jacobs, R.F. 1994. Multiple-drug-resistant tuberculosis. *Clinical Infectious Diseases* 19: 1-10
- Kamath, A. T., Feng, C. G., Macdonald, M., Briscoe, H. & Britton, W. J. 1999. Differential protective efficacy of DNA vaccines expressing secreted proteins of Mycobacterium tuberculosis. *Infection and Immunity* 67: 1702-7.
- Kozak, M. 1986. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acid Research* 15: 8125-48.
- Kumar, H., Malhotra, D., Goswami, S. & Bamezail, R. N. K. 2003. How far have we reached in Tuberculosis vaccine development? *Critical Review in Microbiology* 29: 297-312.
- Launois, P, Deleys, R, Niang, M.N, Drowart, A, Andrien, M, Huygen, K. 1994. T-cell epitope mapping of the major secreted mycobacterial antigen Ag85A in tuberculosis and leprosy. *Infection and Immunity* 62: 3679-3687.
- Lauzardo, M. & Ashkin, D. 2000. Phthisiology at the dawn of a new century. *American College of Chest Physician* 117: 1455-73.
- Lee, B. Y. & Marcus, A. H. 1999. T-cell epitopes mapping of the three most abundant extracellular proteins of Mycobacterium tuberculosis in outbred guinea pigs. *Infection and Immunity* 67: 2665-2670.
- Lowrie, D. B., Tasco, R. E. & Bonato, V. L. D. 1999. Therapy of tuberculosis in mice by DNA vaccination. *Nature* 400: 269-71.
- Mauricio, C.-A., Yolanda, L.-V., Clara, E.-P. & Rogelio, H. 2005. A new vaccine against tuberculosis show the greater protection in a mouse model with progressive pulmonary tuberculosis. *Tuberculosis* 85: 115-126.
- Norazmi, N.M, Mustaffa, M. 2004. Approaches towards the development of vaccine against Tuberculosis : recombinant BCG and DNA vaccine. *Tuberculosis* 84: 102-109.
- Sambrook, J. & Rusell, D. W. 2001. Molecular cloning: a

laboratory manual, Cold Spring Harbour Laboratory Press, N Y.

Silva, C. L., Bonato, V. L. D., Lima, K. M., Coelho-Castelo, A. A. M., Faccioli, L. H., Sartori, A., De Souza, A. O. & Leao, S. C. 2001. Cytotoxic T cells and mycobacteria. *FEMS Microbiology Letters* 197: 11-18.

Stemmer, W. P. C., Cramer, A., Ha, K. D., Brennan, T. M. & Heynecker, H. L. 1995. Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. *Gene* 164: 49-53.

Sterne, J.A, Rodrigues, L.C, Guedes, I.N. 1998. Does the efficacy of BCG decline with time since vaccination? *International Journal of Tuberculosis and Lung Disease* 2: 200-207

Ulmer, J. B., Wahren, B. & Liu, M. A. 2006. Gene-based vaccines: recent technical and clinical advances. *Trends in Molecular Medicine* 12: 216-222.

Wiker, H. G. & Harboe, M. 1992. The antigen 85 complex: a major secretion product of *Mycobacterium tuberculosis*. *Microbiological Reviews* 56: 648-661.