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Induction of an Antibody Response against *Plasmodium falciparum* F2RIIEBA by Heterologous Prime-boost Immunisation

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Abstrak: Strategi imunisasi *prime-boost* secara heterologus boleh merangsang gerak balas antibodi yang lebih berkesan dan mungkin penting dalam pembangunan vaksin malaria yang lebih baik. Dalam kajian ini, kami telah menunjukkan bahawa imunisasi mencit Balb/c dengan vaksin Bacille Calmette-Guerin rekombinan (rBCG) yang mengandungi plasmid yang mengekodkan fragmen sintetik epitop ESAT-6 *Mycobacterium tuberculosis*, fragmen 2 kawasan II antigen pengikat eritrosit (F2RIIEBA) dan tiga jujukan berulang protein circumsporozoite $(NANP)_3$ *Plasmodium falciparum* sebelum diberikan dos penggalak, sama ada dua dos klon rBCG atau vaksin DNA yang mengekspreskan F2RIIEBA natif, menghasilkan paras antibodi anti-F2RIIEBA yang lebih tinggi dalam serum berbanding dengan kaedah imunisasi secara homologous menggunakan dua dos rBCG. Keputusan ini menunjukkan potensi vaksinasi vaksin DNA dalam merangsang gerak balas antibodi terhadap vaksin rekombinan yang mengekspreskan beberapa epitop.

Kata kunci: Malaria, *Plasmodium falciparum*, *Prime-boost*, Vaksin

Abstract: Heterologous prime-boost immunisation strategies can evoke powerful antibody responses and may be of value in developing an improved malaria vaccine. Herein, we show that an immunisation protocol that primes Balb/c mice with a recombinant Bacille Calmette-Guérin (rBCG) vaccine consisting of a plasmid encoding a synthetic fragment of the ESAT-6 epitope of *Mycobacterium tuberculosis*, the fragment 2 region II of erythrocyte-binding antigen (F2RIIEBA) and the three repeat sequences of the circumsporozoite protein $(NANP)_3$ of *Plasmodium falciparum* before subsequently boosting the mice with either two doses of the rBCG clone or with a DNA vaccine expressing the native form of F2RIIEBA generating higher serum anti-F2RIIEBA antibody levels than an immunisation protocol that calls for a homologous prime-boost with two doses of rBCG. These results demonstrate the potential of DNA vaccination in boosting the antibody response to a recombinant vaccine expressing multiple epitopes.

Keywords: Malaria, *Plasmodium falciparum*, Prime-boost, Vaccine

INTRODUCTION

Malaria is caused by several *Plasmodium* species, including *P. falciparum*, and is considered a major health problem in much of the world, especially the tropics. Although effective treatments are available, these treatments have a high failure rate, particularly in the treatment of drug-resistant parasites (White 2004). Thus, an effective malaria vaccine appears to be the only viable long-term strategy that will prevent infection (Weatherall *et al*. 2002). Various strategies are being used

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to develop vaccines against malaria. As a result, a wide variety of malaria vaccine candidates, including an attenuated vaccine (Hafalla *et al*. 2006), a subunit vaccine (Valderrama-Aguirre *et al*. 2005), a DNA vaccine (Wang *et al*. 2005) and a recombinant vaccine (Wu *et al*. 2006), have been developed and tested. However, only some of these vaccine candidates have shown encouraging results in human clinical trials (Sacarlal *et al*. 2008; Alonso *et al*. 2004), with most of the vaccines generating only partial protection against the parasite in mouse models and humans. Thus, it is critical to improve malaria vaccines to a point where these treatments can generate long lasting immunity and protective efficacy.

Prime-boost vaccination has emerged as a highly effective strategy in generating long-lasting immune responses and protective efficacy against diverse pathogens, such as malaria (Walther *et al*. 2006; Dunachie & Hill 2003), HIV (Brown *et al*. 2010; Pal *et al*. 2006), and tuberculosis (Kaufmann 2006; Mc Shane *et al*. 2001), and against cancer (Lin *et al*. 2003). The prime-boost strategy utilises two different vaccines that express the same antigen but have different modes of delivery. In this method, subjects are first primed with one agent (e.g., a DNA vaccine) before receiving a different agent (such as a recombinant pathogen or protein) as a booster; this strategy enhances the immune response or protective efficacy of the initial vaccine. Although the actual mechanism by which this strategy induces robust immune responses against the antigen remains unclear, it has been speculated that the features of the vectors used in this strategy are responsible for these outcomes (Woodland 2004). The first vaccine stimulates the production of memory cells, and the second vaccine expands the memory response that was generated by the first immunisation. Thus, this strategy may stimulate different types of immune responses against the antigen (Dunachie & Hill 2003).

The effectiveness of prime-boost immunisation strategies in the development of a malaria vaccine has been proven in animals and humans; however, most studies have demonstrated that superior levels of immunity are achieved if DNA vaccines are used to prime the immune response and then recombinant vaccines or proteins are used as boosters. Schneider *et al*. (1998) showed that DNA priming and modified vaccinia virus Ankara (MVA) boosting provided sterilising immunity, while MVA priming/DNA boosting was ineffective. Hill and colleagues (Hill *et al*. 2000) showed that a prime-boost immunisation strategy utilising a DNA vaccine and recombinant MVA successfully induced T cell responses in both mice and non-human primates. Furthermore, a prime-boost strategy utilising DNA as the priming agent and MVA as the boosting agent was shown to induce strong and long-lasting Th1 responses in humans (Vuola *et al*. 2005). Miao and colleagues (Miao *et al*. 2006) also determined that priming with a DNA vaccine expressing *P. falciparum* apical membrane antigen 1 (AMA1) and boosting with a recombinant vaccinia poxvirus expressing either the same epitope (rMVA) or recombinant AMA1 protein (rAMA1) induced a specific immune response in Balb/c mice.

Erythrocyte-binding antigen 175 (EBA-175) is a 175-kDa protein that is expressed in the micronemes of merozoites. EBA-175 binds to the erythrocyte receptor sialic acid on glycophorin A and is involved in the formation of a junction

between the erythrocyte and the merozoite (Sim *et al*. 1990). EBA-175 binds to glycophorin A on the surface of human erythrocytes via the fragment 2 region II of erythrocyte-binding antigen's (F2RIIEBA) N-terminal cysteine-rich region and is a target of antibody responses. Previous studies have shown that antibodies directed against F2RIIEBA block EBA-175 binding to erythrocytes and inhibit parasite growth in vitro (Okenu *et al*. 1997). Furthermore, antibodies against this antigen were also detected in the sera of individuals living in an area of western Kenya where malaria is endemic, suggesting the importance of this antigen in the development of malaria (Ohas *et al*. 2004).

In this study, we constructed two different vaccines: a recombinant Bacille Calmette-Guérin (rBCG) vaccine and a DNA vaccine. The rBCG vaccine contained a plasmid encoding a synthetic fragment consisting of the 6-kDa early secreted antigenic target (ESAT-6) of *Mycobacterium tuberculosis*, F2RIIEBA, a blood-stage epitope of *P. falciparum* and the three repeat sequences of the circumsporozoite protein $(NANP)_3$ [a pre-erythrocytic epitope of *P. falciparum*]. The DNA vaccine contained the native form of F2RIIEBA. The aim of the present study was to compare the levels of anti-F2RIIEBA antibody induced by priming with the rBCG vaccine and boosting with two doses of the rBCG clone to the levels of anti-F2RIIEBA antibody induced by priming with the rBCG vaccine and boosting with the DNA vaccine.

MATERIALS AND METHODS

Construction of the rBCG Vaccine

The rBCG clone used in this study was constructed in our previous study (Rapeah & Norazmi 2006). Briefly, a rBCG clone expressing a synthetic DNA fragment consisting of the malarial epitopes F2RIIEBA and $(NANP)_3$ in addition to the ESAT-6 epitope of *M. tuberculosis* was generated by assembly PCR using the following primers: sense (5'-ATTCTAGAGCGGATCCTGATTGAGCCCGTCGTCGCGGGCAC-3') and antisense (5'-CGGGATCCGCTCTAGAGGTCCTGGTCGCAGATCT-3'). The amplified fragment was cloned into pCR®2.1-TOPO® (Invitrogen, New York). This plasmid was then converted into a shuttle plasmid through the insertion of the mycobacterial origin of replication (*Myco* ORI) derived from the *Mycobacterium fortuitum* plasmid pAL5000 (Labidi *et al*. 1985) at the *Kpn*1 site. The resulting plasmid was named pNMN002 and transformed into BCG Pasteur by electroporation.

Construction of the DNA Vaccine

The native DNA fragment of F2RIIEBA was amplified from the cDNA of *P. falciparum* CAMP strain (Malaysia) using the following primers: 5'-GGATCCGAAAAGCGTGAACATATT-3' and 5'-GAATTCCGCGAAGTTTGTTCATTATT-3'. The PCR product was digested with *Bam*HI and *Eco*RI and cloned into the DNA vaccine vector pVAX1 (Invitrogen, New York) to produce pNMN004.

Vaccination of Mice

The experimental protocol used in this study was approved by the Animal Ethics Committee of Universiti Sains Malaysia (AECUSM) [No. USM/Animal Ethics Approval/2007/(31)(099)]. Groups of five female BALB/c (H-2^d) mice (5–6 weeks old) were used in all of the experiments. For rBCG-rBCG-rBCG immunisation, the mice received an intraperitoneal (i.p.) injection of 10^6 cfu BCG in 200 μl phosphate buffered saline (PBS) containing 0.1% Tween 80 (PBS-T80). Two control groups of mice were injected with either 10^6 cfu of the parent BCG strain in 200 μl PBS-T80 or with 200 μl PBS-T80. After 30 and 60 days, the same amount of rBCG, parent BCG or PBS-T80 was injected i.p. as a booster.

For rBCG-DNA immunisation, the mice received an i.p. injection of 10^6 cfu BCG in 200 μl PBS-T80. Two control groups of mice were injected with either 10^6 cfu of the parent BCG strain in 200 μl PBS-T80 or with 200 μl PBS-T80 alone. After 30 days, 50 μg of pNMN004 was administered intramuscularly (i.m.) into each musculus tibialis as a booster. Serum was collected from the tail vein before each injection and stored at –80°C until use.

Preparation of F2RIIEBA Fusion Protein

The F2RIIEBA fusion protein was prepared in our previous study (Rapeah & Norazmi 2006). Briefly, the native DNA fragment of F2RIIEBA was amplified from the cDNA of the CAMP strain of *P. falciparum* using the following primers: sense (5'-CGGGATCCGGAAAAGCGTGAACATATT-3') and antisense (5'-CGGGAATTCCGAAGTTTGTTCATTATT-3'). The PCR product was cloned into the expression vector pRSET B (Invitrogen, New York) and transformed into the *E. coli* BL21(DE3) pLysS strain for protein expression. The culture was grown overnight in Luria-Bertani (LB) media containing 50 μg/ml ampicillin at 37°C before being diluted 1:10 with fresh media and further incubated until the optical density (OD) at wavelength of 600 nm (OD_{600}) was 0.4. Isopropylthio-β-galactoside (IPTG) was added to a final concentration of 1 mM, and the culture was incubated for an additional 6 h before the cells were harvested and the proteins separated using 10% preparative sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The target protein was excised from the gel, eluted using an electroelution apparatus (Bio-Rad, Hercules, California) and precipitated with four volumes of ice-cold acetone.

Measurement of IgG and IgG Subclasses by ELISA

Flat-bottom 96-well microtiter plates (Nunc, Wiesbaden, Germany) were coated with 100 μl of 2 μg/ml F2RIIEBA protein in carbonate-bicarbonate buffer at 4°C overnight. After incubation, the plates were washed with PBS containing 0.1% Tween 20 (PBS-T20) and blocked with 200 μl blocking solution (Boehringer Mannheim, Germany) for 1 h at 37°C. The plates were washed twice with PBS before 100 μl of serum (pooled from 5 mice) diluted 1:50 with PBS was added to each well. The plates were then incubated for 1 h at 37°C. After washing, horseradish peroxidase-conjugated goat anti-mouse IgG, anti-IgG1, -IgG2a, -IgG2b or -IgG3 (Dako, Tokyo) [diluted 1:4000 with PBS] was added to the wells, and the plates were incubated for 1 h at 37°C. After 3 washes, 100 μl of

2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS®) (Boehringer Mannheim, Germany) was added to each well. The reaction was stopped with 8 M NH_2SO_4 after 30 min, and the OD at 410 nm was measured with a microplate reader (Dynatech Laboratories, Virginia, USA).

Statistical Analysis

All of the experiments were carried out in triplicate and repeated three times. The data are represented as the arithmetic mean ± standard error of the mean (SEM). Comparisons were made between the groups in each immunisation method or the IgG subclasses using one-way ANOVA. The Mann-Whitney U test was used to compare the different immunisation methods and IgG subclasses in the BCG and rBCG groups. The accepted significance level was $p<0.05$.

RESULTS

Total IgG Response to F2RIIEBA

The increases in the total anti-F2RIIEBA IgG levels were significantly higher in the mice immunised with rBCG and boosted with the DNA vaccine ($p<0.05$) than the mice prime-boosted twice with rBCG (Fig. 1).

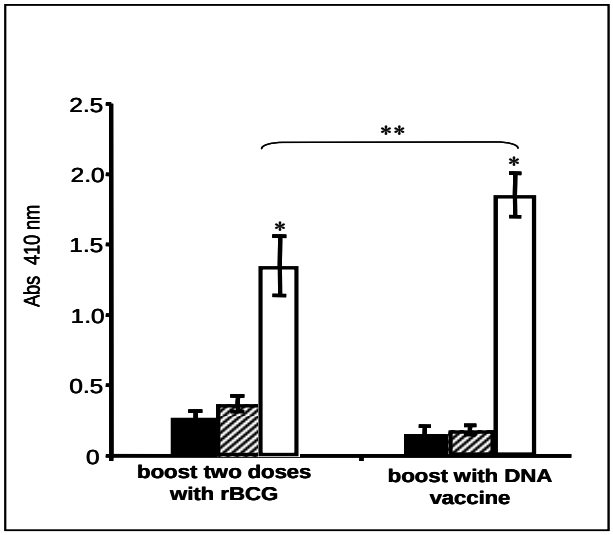
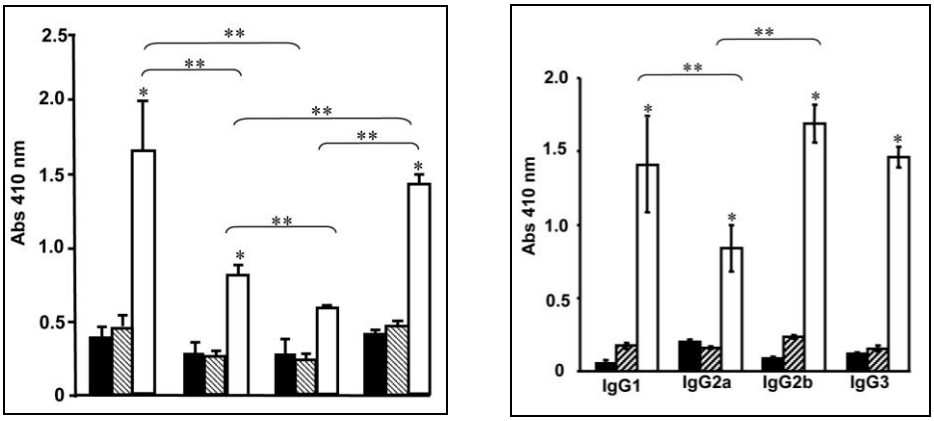


Figure 1: The anti-F2RIIEBA IgG response in Balb/c mice 4 weeks after the last immunisation. PBS-T80 (solid bars), BCG (hatched bars) and rBCG (open bars) were boosted with two doses of the same antigens or the DNA vaccine pNMN004. The antibody levels were measured by ELISA (*, $p<0.05$ between the different groups in each immunisation method; **, $p<0.05$ between the different prime-boost schemes).

IgG Subclass Response to F2RIIEBA

The anti-F2RIIEBA IgG1 levels in the rBCG-rBCG-rBCG immunised mice were higher than the levels of the other antibody subclasses (IgG3 > IgG2a > IgG2b) in the same mice [Fig. 2(a)]. In contrast, the anti-F2RIIEBA IgG2b levels in the rBCG-DNA immunised mice were higher than the IgG1, IgG3 and IgG2a antibody levels (IgG2b > IgG3 > IgG1 > IgG2a) in the same mice [Fig. 2(b)] and higher than the IgG2b levels in the rBCG-rBCG-rBCG immunised mice [Fig. 2(a)].



(a) (b)

Figure 2: The F2RIIEBA-specific IgG subclass response profile in Balb/c mice 4 weeks after the last immunisation, PBS-T80 (solid bars), BCG (hatched bars) and rBCG (open bars): (a) boosted with two doses of the same antigens; (b) boosted with the DNA vaccine pNMN004. The antibody levels were measured by ELISA (*, $p<0.05$ between the different groups in each subclass; **, $p<0.05$ between the different IgG subclasses of the rBCG group).

DISCUSSION

Heterologous prime-boost vaccination using different antigen delivery systems has been used to increase immune responses and achieve long-term protection against various pathogens, including malaria. However, the immunisation sequence and the nature of the antigen delivery systems used in this strategy are important for the induction of immune responses against the antigen. While many vector agents can prime an immune response, not all of these agents are effective at boosting. In many cases, DNA-based vaccines are able to effectively prime an immune response but are relatively ineffective as boosting agents. Recombinant vaccines are more effective at boosting an immune response. In a previous study, the use of a recombinant vaccine for priming and a DNA vaccine for boosting failed to induce specific immune responses against the antigen (Dunachie & Hill 2003). Priming with the first vaccine activates T cells, allowing the development of memory cells. Subsequent boosting with a second vaccine expressing the same antigen(s) will stimulate these cells to undergo rapid

expansion. When the immune system is reactivated, these T cells can then stimulate an enormous expansion of B cells and T cells (Woodland 2004).

Although DNA-based vaccines are inefficient as boosting agents, data have indicated that DNA vaccines can be used as boosting agents under certain conditions (such as priming with a recombinant vaccine). Eo *et al*. (2001) showed that maximal mucosal immunity was achieved when a recombinant viral vector vaccine encoding herpes simplex virus glycoprotein B (rvacgB) was used to prime animals mucosally before a DNA vaccine encoding gB (gBDNA) was used as a mucosal booster. In another study, Doria-Rose *et al*. (2003) also found that priming with gene gun-delivered DNA followed by boosting with vaccinia virus and vaccinia virus priming followed by DNA boosting provided *Macaca nemestrina* with significant protection against the simian/human immunodeficiency virus SHIV89.6P. Similarly, priming neonatal animals with BCG and boosting with a DNA vaccine consisting of a plasmid encoding the mycobacterial proteins Hsp65, Hsp70 and Apa induced protection similar to the protection provided by a prime-boost with DNA/BCG (Skinner *et al*. 2005). Our results also indicate that heterologous prime-boost vaccination schemes that prime with rBCG and boost with a pNMN004 DNA vaccine expressing F2RIIEBA elicit a higher anti-F2RIIEBA antibody response than schemes utilising homologous prime-boost immunisation with two doses of rBCG. This result indicates that a DNA vaccine could be used to boost an immune response that was initially primed using a recombinant vaccine that expresses multiple epitopes. In this strategy, priming with an rBCG vaccine expressing a number of epitopes (including F2RIIEBA) effectively primes the immune response and generates memory cells targeting these epitopes. The F2RIIEBA-specific immune response is then expanded by immunisation with a DNA vaccine encoding F2RIIEBA.

The two different prime-boost schemes investigated in this study also stimulated the production of different IgG subclasses. Priming with rBCG and boosting with the DNA vaccine predominantly induced the production of IgG1, IgG2b and IgG3. In contrast, priming and boosting with two doses of the rBCG vaccine stimulated the production of higher levels of the IgG1 and IgG3 subclasses but lower levels of the IgG2b subclass. However, both strategies produced IgG2a subclass antibody levels that were lower than the levels of the other subclasses (Fig. 2). This result indicates that different antibody subsets were stimulated by the different immunisation strategies. While both prime-boost strategies produced mixed Th1/Th2 antibody responses, the presence of higher antigen-specific IgG2b levels in the sera of mice that were heterologously prime-boosted with rBCG and a DNA vaccine indicated that immunisation with a DNA vaccine expressing F2RIIEBA increased Th1 activity (Bowman & Holt 2001). However, the molecular mechanism driving this preferential generation of the IgG2b isotype is unclear at present. We believe that this phenomenon might result from the different vectors used in the heterologous prime-boost immunisation scheme: different vectors stimulate different lymphocyte subsets, generating different antibody response patterns (Lu 2009; Woodland 2004). In addition to the vectors and the order of vaccine administration, the immune response generated by prime-boost vaccination also depends on factors such as

the dose of the vaccine, the interval between the first and second antigen exposures, and the similarity of the epitopes (Brown *et al*. 2010).

CONCLUSION

In conclusion, previous studies have demonstrated that in heterologous prime-boost regimens, DNA vaccines are excellent priming agents but are relatively ineffective as boosting agents, while recombinant vaccines are capable of either priming or boosting. Our data suggest that the opposite immunisation scheme can also produce an effective immune response if the recombinant vaccine expresses more than one epitope. This preliminary result provides a platform through which the magnitude of the resulting immune response can be increased and the antigenic competition that occurs in vaccines containing multiple antigens or epitopes from the same or different organisms can be reduced.

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