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Functional evaluation of malaria Pfs25 DNA vaccine by *in vivo* electroporation in Olive baboons

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Abstract

Plasmodium falciparum Pfs25 antigen, expressed on the surface of zygotes and ookinetes, is one of the leading targets for the development of a malaria transmission-blocking vaccine (TBV). Our laboratory has been evaluating DNA plasmid based Pfs25 vaccine in mice and non-human primates. Previously, we established that *in vivo* electroporation (EP) delivery is an effective method to improve the immunogenicity of DNA vaccine encoding Pfs25 in mice. In order to optimize the *in vivo* EP procedure and test for its efficacy in more clinically relevant larger animal models, we employed *in vivo* EP to evaluate the immune response and protective efficacy of Pfs25 encoding DNA vaccine in nonhuman primates (Olive baboons, *Papio anubis*). The results showed that at a dose of 2.5 mg DNA vaccine, antibody responses were significantly enhanced with EP as compared to without EP resulting in effective transmission blocking efficiency. Similar immunogenicity enhancing effect of EP was also observed with lower doses (0.5 mg and 1 mg) of DNA plasmids. Further, final boosting with a single dose of recombinant Pfs25 protein resulted in dramatically enhanced antibody titers and significantly increased functional transmission blocking efficiency. Our study suggests priming with DNA vaccine via EP along with protein boost regimen as an effective method to elicit potent immunogenicity of malaria DNA vaccines in nonhuman primates and provides the basis for further evaluation in human volunteers.

Keywords

Pfs25; Malaria; Transmission blocking vaccine; DNA vaccine; Electroporation; Prime boost

1. Introduction

Malaria remains one of the major causes of mortality and morbidity, with 3.2 billion people at risk, 300–500 million clinical cases and more than 1 million deaths annually [1]. The

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malaria parasite has a complex life cycle and various stages provide potential targets for the development of vaccines. In particular, vaccines that target antigens found on sexual and mosquito midgut stages of the parasite are uniquely capable of interrupting malaria transmission [2–4]. Transmission blocking vaccines (TBV) elicit antibodies which, upon ingestion, block parasite development in the mosquito midgut thus rendering them transmission incompetent. TBV candidates in various stages of development for *P. falciparum* include proteins Pfs48/45, Pfs230 and Pfs25 [5–8]. Among them, Pfs25 expressed on the surface of gametes, zygotes and ookinetes, is one of the most investigated targets for TBV development. Pfs25 has been difficult to produce in reproducibly functional conformation in recombinant expression systems [10, 11]. Despite these challenges, considerable progress has been made in the expression and enhancement of immunogenicity along with formulation in various adjuvants [12–14]. A phase I clinical trial of Pfs25 expressed in *Pichia pastoris* formulated with Montanide ISA 51 revealed moderate immunogenicity [15] emphasizing the need for improved vaccine design and alternate approaches.

We have been investigating Pfs25 in the form of DNA vaccine plasmids [16–19] as an alternate approach. The rationale for DNA based vaccine development has been to exploit the mammalian host's cellular machinery to produce the protein antigen for presentation to the host immune system [20]. In previous studies in mice, a DNA vaccine expressing Pfs25 elicited strong antibody responses [16], while delivery by *in vivo* electroporation (EP) significantly enhanced immunogenicity [19]. The EP has been used for over 20 years as a means of introducing macromolecules, including DNA into cells *in vitro* [21] and for transfection of plasmids into different tissues *in vivo* [22]. *In vivo* EP is believed to increase the immunogenicity of DNA vaccines via recruitment of immune cells such as dendritic cells, T and B lymphocytes to the site of immunization [25, 26]. Encouraged by enhanced immunogenicity of Pfs25 DNA vaccine by EP in mice, we evaluated EP delivery of Pfs25 DNA vaccine in nonhuman primates (Olive baboons) for the development of a potential transmission blocking vaccine against *P. falciparum*.

2. Materials & Methods

2.1. DNA vaccine plasmids

Pfs25 sequence optimized for expression in mammalian cells [18], lacking signal and anchor sequences was cloned in VR1020 (Vical, Inc., San Diego, CA). Additionally, all the three putative N-glycosylation sites were mutated to replace N residues with Q. Plasmid DNA (<30 EU per mg endotoxin) was purified by Aldevron (ND, USA) and supplied at a concentration of 2.5 mg/ml.

2.2. Immunizations of Olive baboons (*Papio anubis*)

The immunization studies were approved by the scientific and ethics review committee of the Institute of Primate Research, (Karen) Kenya (#10-10-2007). The baboons were trapped from their wild habitats, maintained in quarantine for 3 months, screened for the presence of infection with worms and protozoan parasites and successfully treated using ivermectin and/or metronidazole as appropriate months before the start of immunization. All the animals (7.0 to 12.4 kg) were free of mycobacterial infection. Detailed hematological tests certified all 20 animals during their quarantine period, just prior to and at the termination of the vaccine trial in excellent health with no discernible side effects.

The animals (N=4) assigned randomly to five groups were sedated with ketamine (10 mg/kg) for immunization with DNA vaccines in endotoxin-free PBS (0.5 ml each site by IM route, quadriceps (Fig. 1). In groups 1, 3, 4 and 5, DNA was administered with *in vivo* electroporation (EP) using an ICHOR pulse generator and TriGrid Electrode arrays (8mm/

15.5mm/7.5mm), Ichor Medical Systems Inc. (San Diego, CA). Animals in groups 1, 2 and 5 received a final boost of recombinant Pfs25 protein (17 ug) emulsified with Montanide ISA51 (total volume 0.5 ml, IM, quadriceps, 2 sites) at 20 week time point (8 weeks after last DNA vaccine immunization).

2.3 Assessment of immunogenicity by ELISA

Baboon sera were analyzed for antibody titers by ELISA using 96-well Immunolon-2 plates coated with 1.5 µg/ml rPfs25 (codon harmonized sequence expressed as His-tagged protein using pET (K-) expression vector in *E. coli*, Kumar et al., in preparation) [19]. HRP-conjugated anti-human IgG (KPL Inc., MD) was used at 1:10,000 dilution and the plates were developed with ABTS substrate at 25°C and read at 405 nm using an ELISA reader (VersaMax, Molecular devices, CA). To determine avidity of antibodies, plates were incubated for 15 min with NaSCN (1, 2, 4, 8M) after primary antibody incubation followed by all the other ELISA steps. The binding of antibody to antigen in the absence of NaSCN was considered as 100% (total binding) and residual binding after NaSCN step was expressed as percent of total binding.

2.4 Western blot analysis

Lysate of gametes purified from *P. falciparum* gametocytes (NF54) was fractioned by 12.5% SDS-PAGE, transferred to nitrocellulose membrane and analyzed using pooled baboon sera (1:5,000). Peroxidase conjugated anti-human IgG (1:10,000) was used as a secondary antibody and the membranes were developed using ECL western blotting detection reagent (GE Healthcare Ltd, UK).

2.5. Membrane feeding assay (MFA)

For MFA, baboon sera were mixed with normal human serum, *P. falciparum* (NF54) gametocytes (0.3% final) and human erythrocytes (50% heamatocrit). MFA with baboon sera were performed as described [19]. Adult (4–5 days old) *Anopheles gambiae* (Keele strain developed by Hillary Hurd and Paul Taylor) mosquitoes starved for 5 hours were allowed to feed through a parafilm using water jacketed glass feeders warmed to 37°C. After 15 minutes, blood fed mosquitoes were maintained for 8–10 days in the insectary (26°C, 70–80% RH). Midgut oocysts were enumerated and mosquito infectivity was measured by comparing oocyst burden as well as prevalence of infected mosquitoes.

2.6. Assessment of transmission blocking activity using mice infected with Pfs25TrPb parasites

The transmission blocking activity of baboon sera was also assessed using *in vivo* transmission of malaria parasites from mice infected with a transgenic *P. berghei* parasite that expresses Pfs25 (Pfs25TrPb) [29] after passive immunization. Briefly, Swiss Webster female mice (5–8 weeks old) were infected with 10⁶ Pfs25TrPb parasites. Four days post-infection, starved *Anopheles stephensi* mosquitoes were allowed to take a blood meal on the mice. Mice were then given either 200 µl pre-immune (n=2) or immune sera (n=4, groups 2 and 5) via I.V. injection, rested for 15 min to allow equilibration of the serum. Starved mosquitoes from the same batch were allowed to feed on these mice. Unfed mosquitoes were removed and blood-fed mosquitoes were maintained in the insectary (20°C, 70–80% RH). Ten days post blood feeding mosquito midguts were dissected and oocysts were enumerated. Oocyst counts in the mosquitoes fed on a given mouse before and after administration of sera were used to calculate percent transmission reduction.

2.7. Statistical analysis

Antibody endpoint titers were defined as serum dilutions giving an absorbance higher than the average absorbance at 405 nm of pre-immune serum + 2SD. The differences in antibody response were analyzed by one way analysis of variance (ANOVA). Percent transmission blocking activity was determined by the formula $100 \times (\text{oocyst number in pre-immune sera} - \text{oocyst number in immune sera}) / \text{oocyst number in pre-immune sera}$. To account for animal to animal variation, pre-immune sera used were from corresponding immunized animals. The prevalence of mosquito infectivity was analyzed by $100 \times (\text{number of mosquito infected} / \text{total number of mosquito dissected})$. Statistical significance of oocyst counts between pre-immune and immune groups was analyzed by the Kruskal-Wallis test. Mann-Whitney U test was used to examine the difference in oocyst counts per mosquito before and after administration of sera in *in vivo* blocking assay. Statistical analysis was performed using GraphPad Instat3 software package and *p* values less than 0.05 were considered significant.

3. Results

3.1 Evaluation of DNA vaccine dose-dependent antibody response with or without *in vivo* EP

The studies reported here were aimed at evaluation of efficacy of DNA vaccine along with *in vivo* EP in non-human primates (Olive baboons). After a single dose immunization, only one out of four animals in group 2 (2.5 mg without EP) exhibited detectable anti-Pfs25 response, while all the animals in group 3, 4 and 5 immunized with varying doses of DNA vaccines (0.5, 1.0 and 2.5 mg, respectively) with EP were positive for anti-Pfs25 antibodies (Fig. 2A, 2B). In comparison to group 2, animals in group 5 given the same dose 2.5 mg, but with EP, elicited much higher antibody response. After administration of an additional 2 doses of the DNA vaccines, anti-Pfs25 antibody titers were 4-fold higher (titer 1:80,000) in group 5 (2.5 mg with EP, Fig. 2D) than in group 2 (2.5 mg without EP, titer 1:20,000, Fig. 2C). These results also revealed comparable anti-Pfs25 ELISA titers after 3 dose immunization between group 2 (2.5 mg without EP) and group 3 (0.5 mg with EP) indicating a minimum of 5-fold dose sparing effect of the EP method (Fig. 2B). No anti-Pfs25 antibodies were detected in the control groups (Group 1). These results clearly demonstrate that EP enhanced the immunogenicity of the Pfs25 DNA vaccine, while immune response without EP at relatively high antigen dose were lower and variable from animal to animal.

3.2. Recombinant protein boost enhances the potency of DNA vaccines

Animals in group 1 (vector alone), group 2 (2.5mg without EP) and group 5 (2.5 mg with EP) were given a final boost with Pfs25 emulsified in Montanide ISA51. After protein boost, animals in both the groups (2 and 5) showed much higher anti-Pfs25 antibody titers (mean reciprocal titers exceeding 1:640,000) as compared to vector alone group 1 (1:1600) (Fig. 2E). These results suggest that immunization with DNA alone, with or without EP, was effective in priming antigen-specific immune response, and a single dose of heterologous protein antigen delivery resulted in robust boosting of antibody responses in both the groups.

3.3. Avidity of antibodies and recognition of native protein

A method based on dissociation of antibody and antigen by NaSCN was employed to measure avidity/strength of binding. The concentration of NaSCN to effect 50% dissociation of bound antibody was approximately 4M with sera after the protein boost (Fig. 3C, D) as compared to less than 2.3M for sera after 3 DNA immunizations regardless of EP and prior to protein boost (Fig 3A, B). These differences suggest that EP did not affect avidity of antibodies, however, protein boost resulted in antibodies of higher avidity. Western blotting

results revealed strong recognition of Pfs25 in the parasite lysates by antibodies in the sera from groups 2, 3, 4 and 5, while sera from group 1 and pre-immune sera from all the groups did not show any reactivity (Fig. 4)

3.4. Determination of transmission blocking activity by MFA

Transmission blocking assays performed with individual baboon sera at 1:2 dilutions (Table 1) revealed 91% inhibition of oocyst development with sera from baboons immunized with 2.5 mg Pfs25 DNA vaccine with EP. In contrast, sera from baboons immunized with the same dose of Pfs25 DNA without EP showed only 50% reduction in oocyst number. Sera from vector alone group also demonstrated non-specific inhibition (23%) in the number of oocyst (likely due to non-specific baboon serum factors affecting *P. falciparum* gametocytes in the vectors). Oocyst numbers in the midgut in group 1 and group 2 were not different statistically. Sera from groups 3 (0.5 mg with EP) and 4 (1.0 mg with EP) did reveal reduction in the oocyst number (50% and 86%, respectively). An important observation made from these functional assays was that DNA vaccine dose of 0.5 mg given by EP was as effective as a five-fold higher DNA dose administered without EP. These results not only confirm immune enhancement effect of EP but also provide evidence of a dose-sparing effect with low doses of DNA with EP displaying transmission blocking comparable to >5 times higher DNA dose without EP. After a single protein boost, sera from baboons in groups 2 and 5 effectively inhibited the oocyst development (100% in both groups 2 and 5), and the prevalence of mosquito infectivity was 53% (group 2) and 15% (group 5). Sera from group 1 (control) did not inhibit mosquito infectivity. These results further demonstrate the contribution of both EP immunization and heterologous protein boost to achieve functionally effective anti-Pfs25 antibody titers.

3.5. In vivo transmission blocking assessment

Baboon sera were also evaluated using a murine (*P. berghei*) transgenic parasites (Pfs25trPb) expressing Pfs25 protein [29] model and results compared with those with standard MFA (above). Fig. 5 shows pooled results for sera from four baboons per vaccine group tested individually. Sera from animals immunized (with or without EP) with DNA vaccine encoding Pfs25 revealed strong transmission reduction using the *in vivo* model. These results are in agreement with those with MFA except that EP group had shown slightly higher blocking activity in MFA. In agreement with MFA data, a protein boost resulted in further improved transmission reduction in both groups (with or without EP).

3.6 Reactogenicity and toxicity of vaccination in Olive baboons

All monkeys maintained good body condition and agility throughout, body temperatures were within clinical ranges (36.6–39°C) and their body weights had slight variations from the baseline weights, though the differences were not statistically significant. Most baboons did not experience any muscle transient induration, granulomas, abscesses, ulcers, cutaneous erythema and skin swelling at the injection/vaccination sites. Animals in group 2 (no EP) showed inguinal lymphadenopathy ipsilateral with the immunization but it receded with time. There were no other clinical adverse signs and when necessary iron supplements were administered to animals in all the 5 groups to maintain normal haematocrits. Thus DNA vaccines delivered via EP in the olive baboon were safe and well tolerated.

4. Discussion

Despite the undisputed evidence that Pfs25 is an effective candidate for a vaccine to block malaria transmission by mosquitoes, studies with adjuvanted recombinant Pfs25 expressed in *Pichia pastoris* have shown measurable but weak immunogenicity in healthy volunteers [13, 30, 31]. Reasons for poor immunogenicity are not obvious, however, it is believed that

conformational folding of protein is critical for functionally effective immunogenicity. Production of a correctly folded recombinant protein still remains a major challenge. Our laboratory has focused on a DNA vaccine based approach for Pfs25 with stronger immunogenicity as an alternative to recombinant protein expression. A successful malaria TBV is considered to play critical roles in the global malaria eliminations and eradication goal.

Evaluation of DNA vaccines encoding Pfs25 and Pvs25 (a *P. vivax* homologue) had revealed potent immunogenicity in mice [16, 32], however in non-human primates, DNA vaccines required a heterologous protein boost for functionally effective immunogenicity [18]. In vivo EP method of delivery resulted in 2-log higher immunogenicity of DNA vaccine in mice [19]. The studies presented here revealed significantly improved immunogenicity of Pfs25 DNA vaccine by EP method of delivery in baboons. Comparison of EP to conventional I.M. delivery indicated that EP resulted in 4-fold higher antibody titers. EP delivery induced anti-Pfs25 responses after fewer immunizations, even at lower DNA doses, compared to higher doses of IM injection alone, suggesting that EP delivery of Pfs25 DNA vaccine may enable favorable reductions in the DNA dose and or the number of immunizations required to achieve target level of immune response.

DNA vaccines have exhibited sub-optimal immunogenicity in non-human primate models [33,] and in human clinical trials [34], highlighting the need for more effective immune enhancement strategies. Delivery of DNA vaccine by EP and prime-boost immunization strategy have revealed encouraging results [18, 19]. In our study, priming with DNA vaccines with or without EP followed by protein boosting resulted in significantly increased antibody responses (1:640,000) and enhanced protection. Vaccination via EP followed by boosting with protein showed potent efficacy in the inhibition of oocyst development (100%) and reduction in mosquito infectivity. Superior immunogenicity by EP method was further corroborated by a five-fold-dose sparing effect in terms of antibody titers as well as transmission blocking results. Antibodies produced in response to DNA vaccination recognized native Pfs25 in the parasites and support that Pfs25 protein molecules expressed in the mammalian cells were likely to be in their functional native conformation. Our results do emphasize rather drastic effects on antibody titers correlating with higher avidity of antibodies and enhanced functional activity in *ex vivo* MFA and *in vivo* assays employed.

Though *ex vivo* MFA is the most commonly used assay to assess transmission blocking activity, results presented in this study provide strong support for using the *in vivo* murine model [29] in future studies. Infected mice receiving anti-Pfs25 antibodies showed profound inhibition of the development of oocysts, which was comparable in both the groups without EP (92%) and with EP (83%) in DNA vaccinations, while single protein boost improved the transmission blocking efficacy with EP (100 %) and without EP (95%). Comparison of results in the MFA and in *in vivo* model did reveal some discrepancies. For example MFA revealed higher activity of sera from the EP groups, the murine model did not show such difference. While we do not know the reasons, it could result from quantitative differences in the actual amount of antibodies available for blocking in the MFA versus from direct feeding on mice infected with Pfs25TrPb and passively immunized with immune baboon prior to mosquito transmission.

Evaluation of human malaria vaccines requires experimental animal models that mimic malaria disease comparable to humans [35]. Baboons share similarities with humans that include a 28-day menstrual cycle, a flat discoid placenta and hormonal profiles allowing for malaria in pregnancy studies, and their large body size allowing frequent sampling for any clinical observation [36–38]. They are abundant and are not among the endangered NHPs in Kenya. Additionally, they are susceptible to experimental infection with *P. knowlesi* [35,

39–43] and recently we have also reported on the immunogenicity evaluation of *P. falciparum* TBV antigen Pfs48/45 [8]. Baboons also provide an alternate nonhuman primate model for evaluation of human vaccines under development. Our own results using baboons in the present study are highly encouraging and provide a strong rationale for further evaluation of malaria transmission blocking DNA vaccine in humans.

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Highlight

- We evaluated DNA vaccine encoding Pfs25, a malaria vaccine candidate antigen.
- DNA delivery by in vivo electroporation in baboons elicited functional antibodies.
- DNA prime and protein boost further enhanced blocking antibody response.
- We also validated Pfs25TrPb-murine model for future evaluation of antibodies.

Group	DNA Plasmid	Dose	EP (Yes or No)	rPfs25 Protein Boost
1	VR1020 (wt)	2.5 mg	Yes	Yes
2	Pfs25-VR1020	2.5 mg	No	Yes
3	Pfs25-VR1020	0.5 mg	Yes	No
4	Pfs25-VR1020	1.0 mg	Yes	No
5	Pfs25-VR1020	2.5 mg	Yes	Yes

Fig. 1.

Schematic representation of immunization and sera collection schedule. Animals (4 per group) were immunized at indicated time points. Only animals in groups 1, 2 and 5 received a final heterologous boost with recombinant Pfs25 formulated in Montanide ISA-51. Various bleeds identified as B1 to B6 in the study.

Fig. 2.

Evaluation of antibody titers by ELISA in the sera of baboons (n=4 per group). (A) Comparative antibody reactivity in groups 1, 2, 3, 4 and 5 at dilution 1:3200 in the sera 4 weeks after 1st DNA vaccine immunization ($p < 0.0058$) (B1 bleeds) and (B) sera (bleeds B4) obtained 4 weeks after 3rd DNA vaccine immunization ($p < 0.037$) (C) Various bleeds (B1, B2 and B4) of group 2 animals immunized with 2.5 mg VR1020/25 DNA vaccine without EP (D) Various bleeds (B1, B2 and B4) of group 5 animals immunized with 2.5 mg VR1020/25 DNA vaccine with EP (E) Antibody titers after final recombinant Pfs25 protein boost (bleeds B6 of groups 2 and 5). Results shown are the mean absorbance at each serum dilution. The endpoint titers were defined as serum dilutions giving an absorbance values higher than the average absorbance at 405 nm of pre-immune serum + 2SD. Statistically significant differences in immune response between groups were analyzed with one way analysis of variance (ANOVA). The error bars indicate SD.

Fig. 3. Comparative avidity of antibodies in the presence of various concentrations (M) of NaSCN. Results shown are for individual animals immunized with 2.5 mg DNA dose without EP (panels A and C; before and after protein boost, respectively) and with EP (panels B and D; before and after protein boost, respectively).

Fig. 4. Recognition of native Pfs25 protein in *P. falciparum* gametes by Western blot analysis. Reactivity of sera from pooled sera (4 animals in each group) are shown, Lanes 1; preimmune sera obtained prior to immunization, Lanes 2; sera after 3rd DNA immunization (B4), Lanes 3: sera corresponding to B6 bleeds.

Fig. 5.

Evaluation of transmission blocking activity using Pfs25TrPb-murine model. Mice were infected with Pfs25TrPb (10^6 , I/P) parasites. On day 4 mosquitoes were allowed to feed on Pfs25TrPb-infected mice to give a measure of total transmission before (B) passive immunization with various sera. After mosquito infection, 200 μ l of sera from each immunized animal was infected IV and mice rested for 15 min. to allow equilibration of antibodies. New mosquitoes from the same batch were allowed to feed on each mouse after (A) administration of various sera. Top panel shows results on sera before protein boost (B4 bleeds) and the bottom panel shown sera after the protein boost (B6) bleeds. Two pre-immune sera (PB) were tested individually. Likewise sera from group 2(no EP) and group 5 (EP) were tested individually.

Table 1

Determination of transmission blocking activity by MFA in sera from baboons immunized with DNA vaccines followed by protein boost

Serum	Group 1	Group 2	Group 3	Group 4	Group 5
Normal Human	Mean	20	22.4	9.1	19.2
	Median	9	19	4	14.5
	Infected/total mosq.	25/25	11/13	15/18	8/10
Pre-immune	Mean	19.3	11.7	6.8	19.4
	Median	6	11	4	14
	Infected/total mosq.	61/72	34/38	57/68	45/49
B4	Mean	15.4	9.6	4.4	6.5
	Median	7	5.5	2	2
	Infected/total mosq.	61/77	26/34	35/39	29/46
B6	TBA (%) [*]	-	50	50	86
	Mean	17.6	2	NB	NB
	Median	5	0	NB	NB
P-value	Infected/total mosq.	43/61	17/32	NB	NB
	TBA (%)	-	100	-	-
		NS	<0.0001	0.03	0.0007

NB-Not boosted with recombinant protein

NS-Not significant

* TBA(%) was calculated using median oocyst values obtained with pre-immune and immune sera (B4 and B6 bleeds).