A vaccine to prevent transmission of human malaria: A long way to travel on a dusty and often bumpy road

Nirbhay Kumar

Johns Hopkins Malaria Research Institute, Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, 615 N. Wolfe Street, Baltimore, Maryland 21205, USA

The goal for an effective malaria transmission-blocking vaccine (TBV) is to induce immunity against the stages of the parasite that infect mosquitoes so that malaria transmission can be reduced or halted. Malaria transmission is generally spatially confined to an infectious source, thus a TBV used in a community can effectively suppress malaria transmission to others. Antibodies induced by TBVs target antigens on the surface of sexual and mosquito midgut stages of the malaria parasite and antibodies interfere with the development of the parasites in the midgut of the mosquito. Proteins synthesized in the gametocytes (pre-fertilization antigens, in *Plasmodium falciparum*: Pfs230 and Pfs48/45) and in the zygotes-ookinetes (post-fertilization antigens, in *P. falciparum*: Pfs25 and Pfs28) represent some of the key target antigens for the development of TBVs. All the four proteins contain multiple cysteine-rich sequences and the epitopes recognized by transmission-blocking antibodies are reduction-sensitive conformational in nature. The inability to express properly folded proteins has frustrated a protein-based TBV development approach and DNA-based vaccine constructs were envisaged to overcome the conformational problem in recombinant proteins. Indeed studies in mice and monkeys have firmly established the value of DNA-based TBV approach. Although immunogenic in larger animals, delivery of DNA-based TBVs needs to be further optimized to elicit a strong and long lasting functional immune response. This DNA vaccine platform can also facilitate evaluation of a cocktail of pre- and post-fertilization antigens in pre-clinical setting prior to the development of an ideal and effective TBV for clinical trials in human volunteers.

MALARIA continues to remain among the top three infectious organisms (malaria, TB and HIV) affecting billions of people globally. More than a century has passed since the discovery of the causative agent of malaria[1] and the role of mosquito in malaria transmission[1], and an estimated 300–500 million people living in more than 100 countries in various regions of the world currently still suffer from clinical malaria annually. While there are four species of the *Plasmodium* parasites that cause malaria in humans, malaria caused by *P. falciparum* results in an estimated 1 million deaths per year, 90% of which in Africa alone, mostly children under the age of five. Extreme poverty, political instability, deteriorating socioeconomic conditions, environmental degradation, population migrations and inadequate health care infrastructure are just some of the many reasons implicated in our inability to control malaria. While drugs, insecticide-treated bed-nets and other mosquito vector control interventions are used to control or reduce the impact of malaria, widespread prevalence of drug resistance in the parasites and insecticide resistance in the vectors have essentially resulted in the current worsening global malaria situation. A safe, effective and affordable malaria vaccine is expected to provide a long-lasting approach to prevent infection, reduce disease severity, prevent death and interrupt transmission.

The complex life cycle of the malaria parasite is accompanied by extensive developmental changes in the parasites in the vertebrate host and in the mosquito vector that carry out parasite transmission. Successful cyclic transmission process ensures continuous evolution of genetic diversity in the parasite population and the spread of anti-malarial drug resistance. Various asexual and sexual stages of the malaria parasite provide numerous targets for mounting an immunological attack by vaccines. For instance, the sporozoite stages of the parasite inoculated during mosquito blood feeding initiate the infection in the liver and have been shown to be excellent targets for humoral and cellular immunity during their exoerythrocytic life cycle. Asexual stages developing within erythrocyte are associated with clinical malaria including pathogenic effects and death and are targets of protective immune mechanisms mediated by antibodies and a number of cytokines. These asexual stages are also intimately associated with a well-described phenomenon of antigenic variation, which is proving to be a big hurdle toward asexual stage vaccine development. Likewise, erythrocytic sexual stages and mosquito midgut stages of the parasites have been shown to be effective targets of transmission-blocking antibodies.

Based on the effectiveness of vaccination against infectious diseases in the second part of the last century, significant efforts are being made to develop anti-malaria vaccines. Successful vaccines against polio, yellow fever, small pox, measles, etc. not only protect the vaccinated individuals but also protect others in the community by reducing further dissemination of the pathogen. Malaria vaccines have long been the subject of intense research, and although they have yet to be realized clinically, they have become a real technical possibility. The complexity of malaria vaccine development is reflected in the fact that functionally and antigenically different stages of the parasite need to be targeted to achieve a complete protection and parasite transmission reduction. Malaria vaccines currently under development are aimed at both protecting the vaccinated individuals and reducing malaria transmission within the community.

Malaria infection and consequence of partial immunity

Malaria parasites have evolved to complete their life cycle in the human host of diverse genetic background. Successful establishment of various life cycle forms of the parasite during chronic infection is likely regulated by a programmed and coordinated expression of genes (approximately 5300 open reading frames suggested by the genome project) in the various life cycle stages of the parasite[2–4]. This alone begs the question whether immune response against a handful subset of these potentially immunogenic polypeptides can elicit immunity that can effectively stop the infection, prevent clinical malaria and interrupt transmission. Few, if any, individuals develop sterile immunity, nonetheless, individuals repeatedly exposed to infectious mosquito bites over time, gradually acquire partially protective immunity and the acquired immunity persists as long as the individuals are exposed to repeat infections. Natural immunity may display a spectrum of anti-parasite activity and in areas of high transmission such immunity may limit parasite infectivity, be effective in limiting parasite replication thus having an impact on actual parasite burden in the human host and may also effectively limit actual parasite transmission, i.e. infectivity of gametocytes in the mosquitoes and subsequent transmission to other hosts.

While gradual acquisition of natural immunity is indisputable, the mechanisms of stage-specific partially protective immunity in infants, adults and during pregnancy remain rather elusive[5]. This raises a question pertaining to long-term impact of a candidate vaccine that elicits only partially effective immunity: whether partial immunity conferred by a vaccine would benefit or actually deprive those individuals an opportunity to gradually acquire protective natural immunity and make those populations more vulnerable to future malaria attacks? One can argue

that if a partially protective vaccine in some individuals saves lives, then it should be considered a desirable option in the interim, while we continue to strive for improving these 'imperfect' vaccines. Identification of novel antigens, evaluation of various immunostimulatory adjuvants and ways to deliver them for optimum immunogenicity must go on. In the end, a successful malaria vaccine deployment will depend upon actual use of combination of vaccines targeting multiple targets and more likely integrating malaria vaccine programme with other malaria control options, including effective chemotherapy and anti-vector programmes limiting human–vector interactions. An approach that results in actual reduction of malaria transmission can have benefits beyond the direct goal of transmission reduction.

One of the several challenges faced by malaria vaccine developers is dealing with the question of parasite genetic polymorphism reflected in continuous antigenic variation within the parasite surface antigens resulting in evasion of immune response by the parasite[5]. Thus antigenic variation may render vaccines targeting asexual life cycle stages of the parasite ineffective and dilute their overall effectiveness due to increased prevalence of vaccine-resistant parasites in the hosts. One approach would be to combine vaccines targeting various common genotypes of the parasite present in the target host populations. Alternatively, approaches that limit transmission of vaccine-resistant parasites can effectively limit the repertoire of circulating parasite genotypes within the populations. The remainder of this review will focus on the latter vaccine concept, i.e. a malaria transmission-blocking vaccine (TBV)[6–9]. Several excellent reviews have been published updating the concept and pre-clinical and phase I clinical trials with candidate TBVs. This review will particularly focus on some of the more recent vaccine approaches based on the concept of DNA vaccination.

The concept of malaria TBVs

TBVs against malaria are intended to induce immunity against the sexual stages of the parasite that infect mosquitoes so that malaria transmission is reduced or halted. The goal for any effective malaria TBV is to elicit high levels of antibodies which in turn when ingested by mosquitoes will inhibit parasite development in the mosquito midgut (Figure 1). Malaria transmission occurs locally and 'focally', i.e. spatially confined to few hundred meters of an infectious source, thus TBVs used in a community can effectively suppress malaria transmission to others[6–9]. Conceptually, the primary goal of TBV is to prevent or effectively reduce mosquito infectivity and thus reduce transmission. An effective transmission reduction will not only result in reduced mortality and morbidity but also, in some cases, lead to elimination of the parasite in areas of low transmission. In addition, when combined with other

protective vaccines or chemotherapeutic agents, such a vaccine may effectively limit spread of vaccine escape or drug-resistant mutant parasites. In addition, TBV may even help maintain a malaria-free zone in areas where successful parasite elimination is achieved by other malaria control approaches in use. Beginning with the early work of Huff et al.[10] in avian malaria parasite *P. gallinaceum*, the concept of TBV was firmly established by Gwadz[11] and Carter and Chen[12] by immunizing animals with purified sexual stage parasites and assessing malaria transmission to mosquitoes. These studies also demonstrated that the effective immunogenicity was elicited by sexual stage antigens and antibodies alone were sufficient to reduce or prevent parasite infectivity in the mosquitoes, i.e. transmission blocking. Additional role for complement has also been demonstrated for some antibodies.

Just like mosquitoes are essential to malaria transmission, no transmission can occur if the mosquitoes are not infected or do not support the parasite development. Malaria transmission blocking immunity aims to achieve the latter, i.e. to block parasite's development by interfering with the initial stages of development taking place soon after ingestion of infected blood meal (Figures 2 and 3). The stages successfully affected by antigen-specific transmission-blocking immunity include fertilization between male and female gametes or zygotes undergoing transformation into ookinetes.

There is no convenient laboratory animal model for routine evaluation of transmission of *P. falciparum* and *P. vivax*. The efficacy of transmission-blocking antibodies has been evaluated using a mosquito membrane feeding assay (MFA). The MFA involves feeding female anopheline mosquitoes on a mixture of test antibodies and infectious cultured gametocytes (for *P. falciparum*) or gametocytes obtained from an infected person or chimpanzee (for *P. vivax*)[13] and measuring oocyst burden in the mosquito midgut (Figure 2). The MFA, although providing a powerful and valuable tool to evaluate functional activity of

elicited anti-bodies, remains tedious, expensive, cumbersome and often unpredictable.

Further studies using monoclonal antibodies (MAbs) were instrumental in the identification of several antigens in the sexual stages of the human malaria parasite. Such studies have identified two major categories of antigens: those known as pre-fertilization target antigens expressed in the gametocytes (Pfs230 and Pfs48/45 in *P. falciparum*) (Figure 3) and those known as post-fertilization target antigens which are predominantly expressed during zygote to ookinete development of the parasite in the mosquito midgut (Pfs25 and Pfs28 in *P. falciparum*) (Figure 3)[14]. Homologues of many of these antigens have
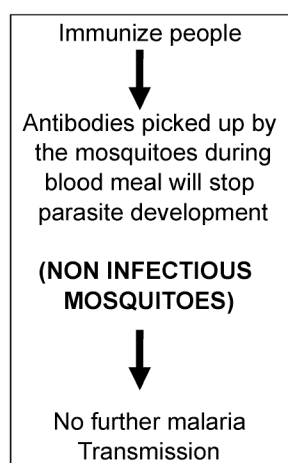

Figure 2. Membrane feeding of mosquitoes.

Figure 1. The concept of malaria transmission-blocking immunity.
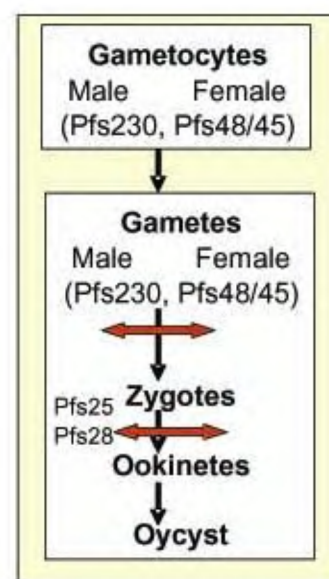
Figure 3. Sexual developmental stages of *Plasmodium* and relevant pre- and post-fertilization target antigens for the development of transmission-blocking vaccines.

also been identified in other species of *Plasmodium*, including *P. vivax*, *P. gallinaceum* (avian parasite), *P. berghei* and *P. yoelii* (rodent parasites)[6,15].

Pre-fertilization antigens (Pfs230 and Pfs48/45) are expressed while gametocytes circulate in the human host, and are targets of natural immune responses. It has been suggested that immune response against these antigens might even be boosted after natural infection thus maintaining high circulating antibodies. This is an important consideration for focusing vaccine development efforts on these antigens. Humans exposed naturally do mount an immune response and transmission-reducing activity of these sera correlates with the presence of antibodies against Pfs230 and Pfs48/45. In sharp contrast, post-fertilization antigens (Pfs25 and Pfs28) are expressed only during mosquito stage parasite development. As one would predict these antigens are not targets of natural immune response elicited during infection, however, immunization studies have amply demonstrated that when appropriately formulated with adjuvants, these antigens are highly immunogenic. The preceding discussion thus makes the important point that immune responses (either naturally elicited during infection or actively induced by vaccination) against pre-fertilization antigens are likely to be boosted during natural infection while no such boosting would occur for post-fertilization antigens (see Kaslow[9]). It is for these reasons it has been a desirable goal to combine these pre-fertilization and post-fertilization antigens in a multivalent, multi-component vaccine to target the transmission stages of the parasite at several points during sporogonic development in the mosquito (double-headed arrows in Figure 3).

Critical requirements for TBV development

Pre-fertilization and post-fertilization antigens have already been identified using specific MAbs and mosquito membrane feeding assay. One of the rather peculiar findings that has emerged is that the target epitopes on these antigens recognized by every single transmission-blocking antibody are relatively cysteine-rich and are disulfide bond-dependent and conformational in nature. Thus it has not been possible to precisely map the target epitopes in all the antigens. This rightaway rules out any synthetic peptide-based vaccine approach at the moment. Thus efforts have largely remained focused in trying to faithfully recreate properly folded recombinant antigens containing the relevant epitopes recognized by transmission-blocking antibodies.

Pre-fertilization target antigens have been rather difficult to express in appropriate conformation. For example, Pfs48/45 has not been expressed at all thus far in a suitable form for evaluation in preclinical trials. Pfs230, on the other hand, is too large to express as a single recombinant product. A smaller fragment (aa: 443–1132) expressed in *E. coli* did induce partially effective antibodies when formulated only in complete Freund's adjuvant and not alum[16]. Given the past and continuing efforts in numerous laboratories, success of recombinant expression of Pfs230 and Pfs48/45 is not predictable, at least not in the near foreseeable future. Further consideration on possible conformational features[17,18] might be helpful in refining and guiding selection of antigenic regions for expression studies. Such fragments containing a proper combination of B and T cell epitope, and if folded properly, might elicit functionally effective transmission-blocking antibodies.

To date most success has been achieved with post-fertilization antigens (Pfs25 and Pvs25, *P. vivax* homologue of Pfs25). Proteins expressed in eukaryotic expression vectors (*S. cerevisiae* or *P. pastoris*) although heterogeneous in nature have reproducibly shown reactivity with the MAbs which recognize conformational epitopes and elicited transmission-blocking antibodies in mice and nonhuman primates. Not too surprising, these proteins expressed in *E. coli*, on the other hand, were not properly folded and did not elicit transmission-blocking antibodies in mice and nonhuman primates. More recently, post-fertilization target antigens expressed in yeast and formulated in alum have undergone limited phase I clinical trial only with limited success[19–22]. There are several reasons for limited immunogenicity in humans. One of the many obvious reasons is that only a small proportion of expressed protein might have been present in native conformation. Future human trials must await further improvement in recombinant expression and formulation using more potent immunomodulatory adjuvants. These post-fertilization antigens have also undergone evaluation as DNA vaccines. As discussed later in this review, immunization with Pfs25-based DNA plasmid in PBS revealed potent immunogenicity in mice and rhesus monkeys[23,24]. Likewise immunization with Pvs25 and Pvs28 DNA vaccines in mice has also revealed impressive and potent immunogenicity[13].

DNA-based immunization

The demonstration that cells can be transfected *in vivo* following injection of DNA led to the concept that immune responses can be induced by injection of plasmid DNA encoding an antigen[25]. These initial observations rapidly developed into a new field of vaccinology based on delivery of naked DNA plasmids encoding a variety of antigens[26–28]. The interest in this novel technology has seen explosive proliferation of efforts and DNA vaccines are quite effective in priming the immune responses and inducing immunological memory. DNA vaccines elicit strong immune responses in mice and the responses vary from antigen to antigen in different hosts. Like other subunit vaccines, the DNA vaccination strategy too needs

further optimization for effective immunogenicity in larger animals and non-human primates.

It has been suggested that immunogenicity of DNA vaccines may largely rely on the built-in adjuvant effect of unmethylated CpG motifs in the context of certain flanking bases present in the plasmid DNA[29]. These motifs which are common in some bacterial and viral genomes but absent in mammalian genomes have a broad range of effects on NK cells, B cells, and APCs such as dendritic cells (DC)[30–32]. The CpG motifs belong to the pathogens associated molecular patterns recognized by specialized 'Toll-like receptors', in particular TLR-9. A few studies have attempted to investigate the role of TLR9 in *in vivo* immunogenicity of DNA vaccines[33–35]. Spies *et al.*[36] found that while IL-12 production by bone marrow derived DCs was affected in TLR-9 KO animals, the CTL response to a model antigen, ovalbumin was however, normal and concluded that TLR9–CpG interactions play only minor role in the efficiency of DNA vaccination. On the other hand, a recent study[37] reported an effect on the magnitude of antibody and cellular immune responses by TLR9 and such effects may vary from one immunogen to another and on the amount and dose schedule of vaccination. These studies do, however, suggest that TLR9-CpG interaction may not be the only mechanism governing Th1 biased immune responses typically induced by DNA vaccines. The current thought is that there might be other cell sensors responding to double-stranded plasmid DNA. The support for such notion also comes from the fact that different TLRs are recognized by single-stranded versus double-stranded RNA molecules[38,39].

Cellular targets of injected DNA include muscle cells, (i.m. immunization) and keratinocytes (epidermal gene gun immunization), however the DNA is also picked up by professional antigen-presenting cells present at the site of injection (DC, Langerhan cells in the skin)[40,41]. Once internalized, DNA interacts most likely with an intracellular receptor, for example, 'Toll-like receptor' (TLR-9) via CpG motifs and triggers production of co-stimulatory cytokines[42]. Interestingly, malarial pigment haemozoin, present in large quantities in the blood stages, has been shown[43] to affect DC maturation and interact with TLR-9. There are significant differences between mouse and rhesus or humans in that TLR-9 is expressed on all DCs in mice, whereas it is expressed only on plasmacytoid, and not myeloid, DCs in humans. Whether this is the reason for poor immunogenicity of DNA vaccines in larger animals remains to be investigated. The elicitation of humoral immune responses requires a coordinated sequence of events that include: antigen acquisition and processing by antigen-presenting cells, presentation of MHC class II associated peptides to CD4 T cells in secondary lymphoid organs (i.e. draining lymphnodes), CD4 T cell activation/proliferation and migration to B cell areas in the lymphoid organs; antigen internalization by B cells; cognate interaction between B cells and antigen-specific CD4 T cells ultimately resulting in clonal B cells expansion and further differentiation into high-affinity antibody producing plasma cells (affinity maturation). Defects in any of these events can lead to defective antibody production in response to vaccination.

Although significant successes have been achieved in laboratory animal species, the level of immunity and protection afforded by DNA vaccines in larger animals and humans is often more limited than by conventional vaccines. One easy explanation could be offered as due to inadequate (compared to body mass) doses of DNA plasmids tested in larger animals. Several approaches have been used to influence the magnitude as well as the type of immune response induced by DNA vaccines[44]. These can be divided into two broad categories, one focusing on the enhancement of antigen expression and the other on the use of immunomodulatory adjuvants. Enhancement of antigen expression has been achieved by using strong promoters, optimization of codon usage, mutations affecting post-translational modifications, increasing uptake of administered DNA and targeting of expressed antigen to specific antigen-presenting cells of the immune system[45–57]. Other immunomodulatory modifications include: genetic (such as plasmid backbone modification with CpG motifs), co-delivery by various routes (intradermal, intramuscular, intranasal) of plasmids encoding various cytokines, chemokines and co-stimulatory molecules[58] and chemicals (such as alum), complexation with cationic lipids (cationic polylactide–co-glycolide polymer, block polymers, monophosphoryl lipid A, the saponin derivative QS21, dimethyldioctadecyl ammonium bromide, etc.) approaches[59–69]. Additionally, use of neutral or cationic polymeric excipients such as, maltodextrin, chitosan has been shown to enhance *in vivo* expression of plasmid encoded antigen and thus potentiate immunogenicity[70,71]. Heterologous prime-boost approach, i.e. priming with DNA vaccine followed by boosting by the same antigen delivered in a different format, for example, live vectors, recombinant proteins, etc.[72] has consistently revealed potent immunogenicity of DNA vaccines and as such continues to be explored for various DNA vaccines currently under development.

The use of cationic lipid formulation of DNA vaccines has shown specially marked improvement in the overall immunogenicity of DNA vaccines. Of particular interest are cationic lipid formulations which are undergoing pre-clinical trials[73–78]. Anthrax, tuberculosis and rabies DNA vaccine plasmids formulated in cationic lipids (Vaxfectin and DMRIE/DOPE) elicited strong immune responses in test animals (rabbits, mice and horses)[79–81]. In a recent study, Cai *et al.*[82] reported on significantly improved antibody levels as well as protective immunity in cattles immunized with a cocktail of three DNA vaccines in the presence of dimethyldioctadecyl ammonium bromide.

Various ways to deliver the DNA plasmid by means other than needle injection have also been explored[83]. Gene gun-based delivery of DNA vaccines has been shown to

be an effective way to induce immune responses against antigens which are otherwise poorly immunogenic by other routes. Another technology involves delivery of DNA plasmids using microenhancer arrays (MEAs). In this technique, DNA is pipetted onto MEA chips fabricated using isotropic potassium hydroxide etch technique, and DNA soaked pads are moved laterally 5–6 times in alternating directions at the site of topical administration. Using the MEA approach, Mikszta et al.[84] reported significantly enhanced humoral and cellular immunogenicity of DNA vaccines based on hepatitis B surface antigen. Yet another promising approach that has shown consistently improved immunogenicity of DNA encoded antigens is the physical method of electroporation. Otten et al.[85] reported substantial improvement in the potency of HIV DNA vaccine in nonhuman primates, as demonstrated by early onset of cellular and humoral immune responses. In vivo electroporation increases expression of DNA encoded antigens which in turn translates into lowering the amount of DNA to be administered in larger animals on per unit body weight basis. These, in addition to other mechanisms, seem to provide an explanation for improved immunogenicity of DNA plasmids delivered by in vivo electroporation method[86–90].

Recent findings on transmission-blocking DNA vaccine constructs

To evaluate safety, immunogenicity and efficacy of the Pfs25 DNA vaccine in nonhuman primates, rhesus macaques (Macaca mulatta) were immunized with DNA vaccine plasmids (VR-1020) encoding Pfs25, Pfg27-Pfs25 hybrid and empty plasmid alone. Immunization with four doses of DNA vaccine constructs elicited antibody with titers approaching 20,000 (figure 1 in ref. 24). However, antibody titers after four DNA doses were not sufficiently high to reduce parasite's infectivity in membrane feeding assays. Further boosting with recombinant Pfs25 formulated in Montanide ISA-720 increased antibody titres (30–80 fold) and significantly blocked transmission of P. falciparum gametocytes to Anopheles mosquitoes (~90% reduction in oocyst numbers in the midguts)[24]. Transmission-blocking assays also revealed that the blocking activity was inversely correlated with serum dilution and similar results were obtained in two different species of mosquitoes (A. gambiae and A. stephensi). Further characterization of sera revealed that antibodies induced by DNA vaccines recognized native Pfs25 in purified gametes of P. falciparum and IgG1 was the dominant isotype of elicited antibodies. NaSCN (a chaotropic reagent) mediated dissociation of bound antibody revealed that the avidity of antibodies after DNA immunization was higher than that of antibodies elicited by a single protein immunization, and the antibody avidity improved further after the protein boost.

These studies thus demonstrated that Pfs25 DNA vaccine was immunogenic in rhesus monkeys and the anti-

body responses were further boosted by heterologous immunization using recombinant protein (Pfs25). Not only did the protein boost improve antibody responses quantitatively (enhanced antibody titres), but also qualitatively and functionally. Antibodies after the protein boost did appear to undergo further affinity maturation, as revealed by avidity assays and detection of IgG2 and IgG4 isotypes among a dominant IgG1 response. The final distinction was provided by the membrane feeding assays in which antibodies after the protein boost resulted in a dose-dependent inhibition of gametocyte infectivity in mosquitoes. These studies suggest the importance of priming with DNA vaccine as an alternative approach to recombinant protein vaccines in future vaccine trials in humans. Moreover, the DNA vaccines can facilitate delivery of several antigens simultaneously and elicit both cellular and humoral immune responses. This observation provides the strong rationale for combining post-fertilization antigen Pfs25 with pre-fertilization target antigens Pfs230 and Pfs48/45 in a cocktail of DNA vaccines for increased efficacy of transmission-blocking activity.

DNA vaccine constructs encoding P. vivax post-fertilization, zygote/ookinete surface proteins, Pvs25 and Pvs28 have also undergone tests in mice. Antibodies produced in mice after immunization with three doses recognized respective antigens by Western blotting and immunofluorescence assays in the parasites and in an ELISA with high titres (see figures 1 and 2 in ref. 13). In membrane-feeding assays using P. vivax infected blood from chimpanzees (P. vivax parasites unlike P. falciparum cannot be cultured), these antibodies resulted in potent (>90 %) blocking of P. vivax transmission to mosquitoes. Several combinations of homologous and heterologous antigen-delivery prime boost strategy were also evaluated and the results suggested that antibody titres and transmission-blocking activities by the three prime-boost strategies (DNA prime/DNA boost, DNA prime/protein boost, and protein prime/protein boost) were comparable with slightly better immunogenicity of heterologous antigen-delivery prime/boost as compared to DNA/DNA alone. These results demonstrate once again potent and transmission-blocking immunogenicity of DNA vaccines encoding Pvs25 and Pvs28.

Effect of plasmid backbone modification on DNA vaccine vectors

Since the immunogenicity of DNA plasmids relies to a large extent on the presence of CpG motifs as built-in adjuvants, we wished to assess if insertion of additional CpG motifs in the plasmid backbone would improve immunogenicity further. Any improvement would be important especially to address the issue of relatively poor immunogenicity of DNA vaccines in nonhuman primates. Sequences representing CpG ODNs shown to be active

for human cells (K type ODN – 5'AT<u>CG</u>ACTCT<u>CG</u>AG–<u>CG</u>TTCTT<u>CG</u>TT<u>CG</u>TTCTC3', 31bp or D type-ODN-5'GGTGCAT<u>CG</u>ATAG AGGGGTGCAT<u>CG</u>ATACAG-GGGGG3', 45 bp, the immunostimulatory CpG motifs are underlined) were introduced in the wild type and Pfs25 DNA vaccine plasmids. After modification, D type ODN modified plasmid contained three extra CpG core of D ODN and K type ODN modified plasmid contained five extra K type CpG motifs. Immunostimulatory effects of these plasmids on human PBMC with respect to expression of DC markers (CD83–CD40), maturation of DCs, production of IL-6, γIFN, IL-α have been described in detail elsewhere[91]. Mice immunized with D type ODN modified Pfs25 DNA vaccine plasmid yielded enhanced specific antibody titres with approximately 3-fold higher Pfs25-specific IgG2a. These results imply that modification of plasmid backbone by D type CpG motif might enhance the antigen-specific total IgG responses and similar modifications could be even desirable if Th1-type immune responses and IgG2a isotype-switch are expected as outcomes for any successful DNA vaccination. Results of these studies demonstrate that plasmid backbone modification by insertion of additional CpG motifs can improve immunogenicity of DNA vaccines.

Natural malaria infection boosts immunity

An adaptive immune response requires periodic immunological boosting achieved by repeat immunizations, thus maintaining effective levels of protective immunity. Such boosting of *P. falciparum* transmission-blocking immunity elicited by vaccine is even more desirable since the success of such immunization will depend upon maintaining high antibody titres and continuing ability of immunized people to suppress malaria transmission through reduction of infection in the mosquitoes. It is often stated that natural exposure to recurring infection can provide booster antigenic stimulation for vaccine-induced immunity (natural boosting or anamnestic response). This notion is supported by the fact that pre-fertilization antigens expressed in the gametocyte stages, for example, Pfs230 and Pfs48/45 are indeed targets of natural immune response[9], however, a direct experimental evidence for natural boosting has not been possible to obtain thus far. No studies exist in animal models of human malaria infections or in human populations where boosting of malaria transmission-blocking antibody responses elicited by immunization is demonstrated to occur through natural infection. Longitudinal field studies in endemic areas in infected subjects of various age groups is one way to assess natural boosting occurring during subsequent transmission cycles.

In our own studies, we developed a mouse model to test this concept experimentally. Mice were immunized with a DNA vaccine (plasmid VR1020 encoding *P. berg-*

hei pre-fertilization target antigen Pbs48/45) followed by infection with *P. berghei* (ANKA strain 2.34). These results demonstrated that Pb48/45 DNA vaccine-primed immune responses could be boosted during repeat parasite infections and the antibodies recognize Pb48/45 as confirmed by IFA using fixed gametocytes of *P. berghei* and HEK293 cells transfected with Pb48/45 plasmids and by Western blotting[92].

TBV development – still a long, dusty and bumpy road

Limited clinical trials with sexual stage protein vaccines such as post-fertilization antigens Pfs25 and Pvs25 have proved not to be optimal, most likely due to improper conformational folding and heterogeneity of purified recombinant proteins[19–22]. Clinical trials with other sexual stage antigens such as pre-fertilization target antigens Pfs230 and Pfs48/45 have not even been attempted due to inability to express these proteins to date in appropriate conformation. While full length Pfs230 may be too large to express, smaller fragments of Pfs230 have shown only partial transmission-blocking immune responses in preclinical trials. In general, in spite of intense efforts in our own laboratory and several other laboratories, Pfs230 and Pfs48/45 proteins or immunogenic fragments have yet to become available for pre-clinical trials. This has hampered understanding several key issues, all likely to impact the success of an effective TBV containing pre- and post-fertilization target antigens. The end result of course has been our inability to go ahead with preclinical and clinical TBV evaluation of a cocktail of antigens. There are several important questions that need to be addressed systematically in future studies. In order to elicit functional transmission-blocking antibodies, pre-fertilization and post-fertilization antigens need to be expressed in appropriate conformation. Because of the conformational nature of epitopes, it has not been possible to map target epitopes. To date, most efforts have remained focused in trying to faithfully recreate properly folded recombinant antigens containing the relevant B and helper T cell epitopes. Given that recombinant forms of pre- and post-fertilization antigens are either not available or are not optimally folded we believe that DNA vaccine constructs can facilitate an initial investigation of all the major issues stated above. DNA encoding various antigens or desired fragments can be cloned easily, formulated using cationic lipids if desired, combined to produce a cocktail of vaccines, can be tested by alternate delivery methods and can be used to jump-start investigation of an important issue of natural boosting of vaccine-primed immune response during natural infection. An underlying assumption of course is that proteins expressed in mammalian cells after immunization with DNA vaccine plasmids, are likely to be folded more like that in eukaryotic parasite

and thus elicit functionally effective transmission-blocking immune response in experimental animals and ultimately in humans.

1. Desowitz, R. S., Milestones and millstones in the history of malaria. In *Malaria – Molecular and Clinical Aspects* (eds Wahlgren, M. and Perlman, P.), Harwood Academic Publishers, Singapore, 1999, pp. 3–18.
2. Gardner, M. J. *et al.*, Genome sequence of the human malaria parasite *Plasmodium falciparum. Nature*, 2002, **419**, 498–511.
3. Bozdech, Z., Zhu, J., Joachimiak, M. P., Cohen, F. E., Pulliam, B. and DeRisi, J. L., Expression profiling of the schizont and trophozoite stages of *Plasmodium falciparum* with a long-oligonucleotide microarray. *Genome Biol.*, 2003, **4**, R9.
4. LeRoch, K. G. *et al.*, Global analysis of transcript and protein levels across the *Plasmodium falciparum* life cycle. *Genome Res.*, 2004, **14**, 2308–2318.
5. Dzikowski, R., Templeton, T. J. and Deitsch, K., Variant antigen gene expression in malaria. *Cell. Microbiol.*, 2006, **8**, 1371–1381.
6. Carter, R., Mendis, K. N., Miller, L. H. and Saul, A., Malaria transmission-blocking vaccine – how can their development be supported? *Nature Med.*, 2000, **6**, 241–244.
7. Carter, R., Transmission-blocking malaria vaccines. *Vaccine*, 2001, **19**, 2309–2314.
8. Carter, R., Spatial simulation of malaria transmission and its control by malaria transmission-blocking vaccination. *Int. J. Parasitol.*, 2002, **32**, 1617–1624.
9. Kaslow, D. C., Transmission-blocking vaccines. In *Malaria Immunology* (eds Perlman, P. and Troy-Blomberg, M.), Karger Press, Basel, 2002, pp. 287–307.
10. Huff, C. G., Marchbank, D. F. and Shiroishi, T., Changes in infectiousness of malarial gametocytes. II. Analysis of the possible causative factors. *Exp. Parasitol.*, 1956, **7**, 399–417.
11. Gwadz, R. W., Successful immunization against the sexual stages of *Plasmodium gallinaceum. Science*, 1976, **193**, 1150–1151.
12. Carter, R. and Chen, D. H., Malaria transmission blocked by immunization with gametes of the malaria parasite. *Nature*, 1976, **263**, 57–60.
13. Kongkasuriyachai, D., B-Andrews, L., Stowers, A., Collins, W. E., Sullivan, J., Tsuboi, T. and Kumar, N., Potent immunogenicity of DNA vaccines encoding *Plasmodium vivax* transmission-blocking vaccine candidates Pvs25 and Pvs28 – evaluation of homologous and heterologous prime-boost strategy. *Vaccine*, 2004, **22**, 3205–3213.
14. Saurwein, R. W. and Eling, W. M. C., Sexual and sporogonic stage antigens. In *Malaria Immunoogy* (eds Perlman, P. and Troy-Blomberg, M.), Karger Press, Basel, 2002, pp. 188–203.
15. Tachibana, M., Tsuboi, T., Templeton, T. J., Kaneko, O. and Torii, M., Presence of three distinct ookinete surface protein genes, *Pos25*, *Pos28-1* and *Pos 28-2*, in *Plasmodium ovale. Mol. Biochem. Parasitol.*, 2001, **113**, 341–344.
16. Bustamante, P. J., Woodruff, D. C., Oh, J., Keister, D. B., Muratova, O. and Williamson, K. C., Differential ability of specific regions of *Plasmodium falciparum* sexual-stage antigen, Pfs230, to induce malaria transmission-blocking immunity. *Parasite Immunol.*, 2000, **22**, 373–380.
17. Carter, R., Coulson, A., Bhatti, S., Taylor, B. J. and Elliott, J. F., Predicted disulfide-bonded structures for three uniquely related proteins of *Plasmodium falciparum*, Pfs230, Pfs48/45 and Pf12. *Mol. Biochem. Parasitol.*, 1995, **71**, 203–210.
18. Gerloff, D. L., Creasey, A., Maslau, S. and Carter, R., Structural models for the protein family characterized by gamete surface protein Pfs230 of *Plasmodium falciparum. Proc. Natl. Acad. Sci.*, 2005, **102**, 13598–13603.
19. Kaslow, D. C., Bathrust, A. C., Lensen, T., Ponnudurai, T., Barr, P. J. and Keister, D. B., *Saccharomyces cerevisiae* recombinant Pfs25 adsorbed to alum elicit antibodies that block transmission of *Plasmodium falciparum. Infect. Immun.*, 1994, **62**, 5576–5580.
20. Hisaeda, H. *et al.*, Antibodies to malaria vaccine candidate Pvs25 and Pvs28 completely block the ability of *Plasmodium vivax* to infect mosquitoes. *Infect. Immun.*, 2000, **68**, 6618–6623.
21. Hiseada, H., Collins, W. E., Saul, A. and Stowers, A. W., Antibodies to *Plasmodium vivax* transmission-blocking vaccine candidate antigens Pvs25 and Pvs28 do not show synergism. *Vaccine*, 2002, **20**, 763–770.
22. Malkin, E. M. *et al.*, Phase 1 vaccine trial of Pvs25H: a transmission-blocking vaccine for *Plasmodium vivax* malaria. *Vaccine*, 2005, **23**, 3131–3138.
23. Lobo, C. A., Dhar, R. and Kumar, N., Immunization of mice with DNA-based Pfs25 elicits potent malaria transmission-blocking antibodies. *Infect Immun.*, 1999, **67**, 1688–1693.
24. Coban, C., Phillip, M., Keister, D. and Kumar, N., Induction of *Plasmodium falciparum* transmission-blocking antibodies in non-human primates by DNA vaccines – evaluation of various prime-boost strategies. *Infect. Immun.*, 2004, **72**, 253–259.
25. Wolfe, J. A. *et al.*, Direct gene transfer into muscle cells *in vivo. Science*, 1990, **247**, 1465–1468.
26. Huygen, K., Plasmid DNA vaccination. *Microbes Infect.*, 2005, **7**, 932–938.
27. Lemieux , P., Technological advances to increase immunogenicity of DNA vaccines. *Expert Rev. Vaccines*, 2002, **1**, 89–97.
28. Thalhamer, J., Leitner, W., Hammerl, P. and Brtko, J., Designing immune responses with genetic immunization and immunostimulatory DNA sequences. *Endocr. Regulat.*, 2001, **25**, 143–166.
29. Kleinman, D. M., Currie, D., Gursel, I. and Verthrlyi, D., Use of CpG oligonucleotides as immune adjuvants. *Immunol. Rev.*, 2004, **199**, 201–216.
30. Bauer, M., Redecke, V., Ellwart, J. W., Scherer, B., Kremer, J. P., Wagner, H. and Lipford, G. B., Bacterial CpG-DNA triggers activation and maturation of human CD11c-CD123$^+$ dendritic cells. *J. Immunol.*, 2001, **166**, 5000–5007.
31. Krieg, A. M. *et al.*, CpG motifs in bacterial DNA trigger direct B cell activation. *Nature*, 1995, **374**, 546–549.
32. Krieg, A. M., Immune effects and mechanisms of action of CpG motifs. *Vaccine*, 2000, **19**, 618–622.
33. Babuik, S. *et al.*, TLR 9$^{-/-}$ and TLR9$^{+/+}$ mice display similar immune responses to a DNA vaccine. *Immunology*, 2004, **113**, 114–120.
34. Roberts, T. L., Sweet, M. J., Hume, D. A. and Stacey, K. J., Cutting edge: Species-specific TLR9-mediated recognition of CpG and non-CpG phosphorothioate-modified oligonucleotides. *J. Immunol.*, 2005, **174**, 605–608.
35. Hartman, G. *et al.*, CpG oligonucleotides induce strong humoral but only weak CD4$^+$ T cell responses to protein antigens in rhesus macqaques *in vivo. Vaccine*, 2005, **23**, 3310–3317.
36. Spies, B. *et al.*, Vaccination with plasmid DNA activates dendritic cells via toll-like receptors 9 (TLR9) but functions in TLR9-deficient mice. *J. Immunol.*, 2005, **171**, 5908–5912.
37. Tudor, D., Dubuquoy, C., Gaboriau, V., Lefèvre, F., Charley, B. and Riffault, S., TLR9 pathway is involved in adjuvant effects of plasmid DNA-based vaccines. *Vaccine*, 2005, **23**, 1258–1264.
38. Lund, J. M. *et al.*, Recognition of single stranded RNA viruses by toll-like receptor 7. *Proc. Natl. Acad. Sci.*, 2004, **101**, 5598–5603.
39. Alexopoulou, L., Holt, A. C., Medzhitov, R. and Flavell, R. A., Recognition of double-stranded RNA and activation of NF-kB by toll-like receptor 3. *Nature*, 2001, **413**, 732–738.
40. Howarth, M. and Elliot, T., The processing of antigens delivered as DNA vaccines. *Immunol. Rev.*, 2004, **199**, 27–39.
41. Leifert, J. A., Rodriguez-Carreno, M. P., Rodriguez, F. and Whitton, J. L., Targeting plasmid-encoded proteins to the antigen presentation pathways. *Immunol. Rev.*, 2004, **199**, 40–53.

42. Hemmi, H., *et al.*, A toll-like receptor recognizes bacterial DNA. *Nature*, 2000, **408**, 740–745.
43. Coban, C. *et al.*, Toll-like receptor 9-mediated innate immune activation by malaria pigment hemozoin. *J. Exp. Med.*, 2005, **201**, 9–25.
44. Lederman, L., DNA-based vaccines and immunostimulants. *Genet. Eng. News*, 2000, **20**, 17.
45. Liu, W. J., Gao, F. G., Zhao, K. N., Zhao, W., Fernando, G. J. G., Thomas, R., and Frazer, I. H., Codon modified human papilloma type 16 E7 DNA vaccine enhances cytotoxix T-lymphocyte induction and anti-tumor activity. *Virology*, 2002, **391**, 43–52.
46. Liu, W. J., Zhao, K. N., Gao, F. G., Zhao, W., Legett, G. R., Fernando, G. J. P. and Frazer, I. H., Polynucleotide viral vaccines: codon optimization and ubiquitin conjugation enhances prophylactic and therapeutic efficacy. *Vaccine*, 2002, **20**, 862–869.
47. Yadava, A. and Ockenhouse, C., Effect of codon optimization on expression levels of a functionally folded malaria vaccine candidate in prokaryotic and eukaryotic expression systems. *Infect. Immun.*, 2003, **71**, 4961–4969.
48. Cid-Arregui, A., Juarez, V. and Hausen, H. Z., A synthetic E7 gene of human papillomavirus type 16 that yields enhanced expression of the protein in mammalian cells and is useful for DNA immunization studies. *J. Virol.*, 2003, **77**, 4928–4937.
49. Deml, L. *et al.*, Multiple effects of codon usage optimization on expression and immunogenicity of DNA candidate vaccines encoding the human immunodeficiency virus type 1 Gag protein. *J. Virol.*, 2001, **75**, 10991–11001.
50. Corbet, S., Vinner, L., Hougaard, M. D., Brydter, K., Nielsen, H. V., Nielsen, C. and Fomsgaard, A., Construction, biological activity, and immunogenicity of synthetic, envelope DNA vaccines based on a primary, CCR-5tropic, early HIV type 1 isolate (BX08) with human codons. *AIDS Res. Hum. Retroviruses*, 2000, **16**, 1997–2008.
51. Nagata, T., Uchijima, M., Yoshida, A., Kawashima, M. and Koide, Y., Codon optimization effect on translational efficiency of DNA vaccine in mammalian cells: analysis of plasmid DNA encoding a CTL epitope derived from microorganisms. *Biochem. Biophys. Res. Commun.*, 1999, **261**, 445–451.
52. Megde, J. Z. *et al.*, Increased expression and immunogenicity of sequence modified human immunodeficiency virus type I gag gene. *J. Virol.*, 2000, **74**, 2628–2635.
53. Uchijima, M., Yoshida, A., Nagata, T. and Koide, Y., Optimization of codon usage of plasmid DNA vaccine is required for the effective MHC class I-restricted T cell responses against an intracellular bacterium. *J. Immunol.*, 1998, **161**, 5594–5599.
54. Vinner, L., Nielsen, H. V., Bryder, K., Corbet, S., Nielsen, C. and Fomsgaard, A., Gene gun DNA vaccination with rev-independent synthetic HIV-1 gp160 envelope gene using mammalian codons. *Vaccine*, 1999, **17**, 2166–2175.
55. Stratford, R., Douce, G., Zhang-Barber, L., Fairweather, N., Eskola, J. and Dougan, G., Influence of codon usage on the immunogenicity pf a DNA vaccine against tetanus. *Vaccine*, 2001, **19**, 810–815.
56. Gaucher, D. and Chadee, K., Construction and immunogenicity of a codon-optimized *Entamoeba histolytica* gal-lectin-based DNA vaccine. *Vaccine*, 2002, **29**, 3244–3253.
57. Moore, R. A. *et al.*, Intraepithelial DNA immunization with a plasmid encoding a codon optimized COPV E1 gene sequence, but not the wild type gene sequence completely protects against mucosal challenge with infectious COPV in Beagles. *Virology*, 2002, **304**, 451–459.
58. Sheerlink, J-P.Y., Genetic adjuvants for DNA vaccines. *Vaccine*, 2001, **19**, 2647–2656.
59. Denis-Mize, K. S. *et al.*, Mechanisms of increased immunogenicity for DNA-based vaccines adsorbed onto cationic microparticles. *Cell Immunol.*, 2003, **225**, 12–20.
60. Kabanov, A. V., Lemieux, P., Vinogradov, S. and Alakhov, V., Pluronic block copolymers: Novel functional molecules for gene therapy. *Adv. Drug Del. Rev.*, 2002, **54**, 223–233.
61. Kau, R., Rauthan, M. and Vrati, S., Immunogenicity in mice of a cationic microparticle-adsorbed plasmid DNA encoding Japanese encephalitis virus envelope protein. *Vaccine*, 2004, **22**, 2776–2782.
62. Jin, H., Li, Y., Ma, Z., Zhang, F., Xie, Q., Gu, D. and Wang, B., Effect of chemical adjuvants on DNA vaccination. *Vaccine*, 2004, **22**, 2925–2935.
63. Mollenkopf, H. J. *et al.*, Enhanced protective efficacy of a tuberculosis DNA vaccine by adsorption onto cationic PLG microparticles. *Vaccine*, 2004, **22**, 2690–2695.
64. O'Hagan, D. T., Singh, M. and Ulmer, J. B., Microparticles for the delivery of DNA vaccines. *Immunol. Rev.*, 2004, **199**, 191–200.
65. Garzón, M.R. *et al.*, Induction of gp120-specific protective immune responses by genetic vaccination with linear polyethylenimine-plasmid complex. *Vaccine*, 2005, **23**, 1384–1392.
66. Gebhart, C. L., Sriadibhatla, S., Vinogradov, S., Lemieux, P., Alakhov, V. and Kabanov, A.V., Design and formulation of polyplexes based on pluronic-polyethyleneimine conjugates for gene transfer. *Bioconjugate Chem.*, 2002, **13**, 937–944.
67. Gebhart, C. L. and Kabanov, A. V., Evaluation of polyplexes as gene transfer agents. *J. Controlled Release*, 2001, **73**, 401–416.
68. Sasaki, S., Takesita, F., Kin, K-Q., Ishii, N. and Okuda, K., Adjuvant formulations and delivery systems for DNA vaccines. *Methods*, 2003, **31**, 243–254.
69. Yaroslavov, A. A., Sukhishvili, S. A., Obolsky, O. L., Ryaroslavova, E. G., Kabanov, A. V. and Kabanov, V. A., DNA affinity to biological membranes is enhanced due to complexation with hydrphobized polycation. *FEBS Lett.*, 1996, **384**, 177–180.
70. Huang, C. Y. *et al.*, Enhancements in gene expression by the choice of plasmid DNA formulations containing neutral polymeric excipients. *J. Pharmaceut. Sci.*, 2002, **91**, 1371–138.
71. Illum, L., Jabbal-Gill, I., Hinchcliffe, M., Fisher, A. N. and Davis, S. S., Chitosan as a novel nasal delivery system for vaccines. *Adv. Drug Del. Rev.*, 2001, **51**, 81–96.
72. Moore, A. C. and Hill, A. V. S., Progress in DNA-based heterologous prime-boost immunization strategies for malaria. *Immunol. Rev.*, 2004, **199**, 126–143.
73. Locher, D. P., Witt, S. A., Ashlock, B. M. and Levy, J. A., Evaluation of genetic immunization adjuvants to improve the effectiveness of a human immunodeficiency virus Type 2 (HIV-2) envelope DNA vaccine. *DNA Cell Biol.*, 2004, **23**, 107–110.
74. Perrie, Y., Frederik, P. M. and Gregoriadis, G., Liposome-mediated DNA vaccination: the effect of vesicle composition. *Vaccine*, 2001, **19**, 3301–3310.
75. Nukuzuma, C., Ajiro, N., Wheeler, C. J. and Konishi, E., Enhancing effect of Vaxfectin on the ability of a Japanese encephalitis DNA vaccine to induce neutralizing antibody in mice. *Viral Immunol.*, 2003, **16**, 183–189.
76. Reyes, L. *et al.*, Vaxfectin enhances antigen specific antibody titers and maintains Th1 type immune responses to plasmid DNA immunization. *Vaccine*, 2001, **19**, 3778–3786.
77. Hartikka, J. *et al.*, Vaxfectin enhances the humoral immune response to plasmid DNA-encoded antigens. *Vaccine*, 2001, **19**, 1911–1923.
78. Fischer, L., Minke, J., Dufay, N., Baude, P. and Audonnet, J-C., Rabies DNA vaccine in the horse: Strategies to improve serological responses. *Vaccine*, 2003, **21**, 4593–4596.
79. Fischer, L., Tronel, J. P., Minke, J., Barzu, S., Baudu, P. and Audonnet, J. C., Vaccination of puppies with a lipid-formulated plasmid vaccine protects against a severe canine distemper virus challenge. *Vaccine*, 2003, **21**, 1099–1102.
80. D'Souza, S. *et al.*, Improved tuberculosis DNA vaccines by formulation in cationic lipids. *Infect. Immun.*, 2002, **70**, 3681–3688.

81. Harmanson, G. *et al.*, A cationic lipid formulated DNA vaccine confers sustained antibody-mediated protection against aerosolized anthrax spores. *Proc. Natl. Acad. Sci. USA*, 2004, **101**, 13601–13609.

82. Cai, H., Tian, X., Hu, X. D., Ki, S. X., Yu, D. H. and Zhu, Y. X., Combined DNA vaccines formulated either in DDA or saline protect cattle from *Mycobacterium bovis* infection. *Vaccine*, 2005, **23**, 3887–3895.

83. Partidos, C. D., Beignon, A. S., Briand, J. P. and Muller, S., Modulation of immune responses with transcutaneously deliverable adjuvants. *Vaccine*, 2004, **22**, 2385–2390.

84. Mikszta, J., Alarcon, J. B., Brittingham, J. M., Sutter, D. E., Pettis, R. and Harvey, N. G., Improved genetic immunization via micromechanical disruption of skin-barrier function and targeted epidermal delivery. *Nature Med.*, 2002, **8**, 415–419.

85. Otten, G. *et al.*, Enhancement of DNA vaccine potency in rhesus macaques by electroporation. *Vaccine*, 2004, **22**, 2493–2498.

86. Luxembourg, A., Hannaman, D., Ellefsen, B., Nakamura, G. and Bernard, R., Enhancement of immune responses to an HBV DNA vaccine by electroporation. *Vaccine*, 2006, **24**, 4490–4493.

87. Widera, G. *et al.*, Increased DNA vaccine delivery and immunogenicity by electroporation *in vivo*. *J Immunol.*, 2000, **164**, 4635–4640.

88. Zhao, Y-G. *et al.*, Anti-HBV immune responses in rhesus macaques elicited by electroporation mediated DNA vaccination. *Vaccine*, 2006, **24**, 897–903.

89. Selby, M., Goldbexk, C., Pertile, T., Walsh, R. and Ulmer, J., Enhancement of DNA vaccine potency by electroporation *in vivo*. *J. Biotechnol.*, 2000, **83**, 147–152.

90. Babiuk, S., Baca-Estrada, M. E., Foldvari, M., Storms, M., Rabussay, D., Widera, G. and Babiuk, L. A., Electroporation improves the efficacy of DNA vaccines in large animals. *Vaccine*, 2002, **20**, 3399–3408.

91. Coban, C., Ishii, K. J., Gursel, M., Klinman, D. and Kumar, N., Modulation of immunogenicity of DNA plasmids by modification of the backbone by two distinct human CpG motifs. *J. Leukocyte Biol.*, 2005, **78**, 647–655.

92. Haddad, D., Maciel, J. and Kumar, N., Infection with *Plasmodium berghei* boosts antibody responses primed by a DNA vaccine encoding gametocyte antigen Pbs48/45. *Infect. Immun.*, 2006, **74**, 2043–2051.