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Antigens for pre-erythrocytic malaria vaccines: building on success

C. SPEAKE[1,2] and **P. E. DUFFY**[1,2]

[1]Malaria Program, Seattle Biomedical Research Institute, Seattle, WA, USA

[2]Department of Global Health, University of Washington, Seattle, WA, USA

SUMMARY

Immunization with attenuated pre-erythrocytic malaria parasites can confer sterile protection against malaria in humans and rodents, and a single pre-erythrocytic antigen incorporated in a subunit vaccine has substantially reduced clinical *Plasmodium falciparum* malaria episodes in African infants during phase 2 trials. Building upon this success has been hindered by technical obstacles that limit research on pre-erythrocytic parasites, especially the liver stage (LS) parasites, and by an incomplete understanding of the immune mechanisms that confer protection in humans. Recent improvements in growing and isolating LS parasites have allowed progress in defining the transcriptome and proteome of the LS parasite, although more work remains to be done particularly for the early LS parasite of *P. falciparum*. Next generation pre-erythrocytic antigens can be assessed and prioritized based on immunization studies in animals, and on models of immunity such as attenuated parasite vaccines that confer sterile protection or naturally acquired LS-specific immune responses that correlate with protection in endemic areas. Although mechanisms of protection in humans remain poorly understood, the availability of a human malaria challenge model for early clinical testing of candidate vaccines is a valuable tool to confirm which immunogens should move forward to larger field trials.

Keywords

malaria; *Plasmodium*; pre-erythrocytic; vaccines

VACCINES AGAINST PRE-ERYTHROCYTIC PARASITES

The worldwide malaria burden would be reduced by vaccines that either prevent infection or prevent transmission. Vaccines that target the earliest stages of the malaria parasite in the human host have the potential to achieve both goals. The mosquito inoculates sporozoite forms into skin during its blood meal, and within an hour these have traversed vascular endothelium to enter the blood stream and passed through the circulation to the liver where they invade hepatocytes and initiate liver stage (LS) development. Because the sporozoite

Correspondence: Patrick E. Duffy, Malaria Program, Seattle Biomedical Research Institute, Seattle, WA 98109, USA (patrick.duffy@sbri.org).

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and liver stages precede the blood stage when disease and death occur, they are referred to as the pre-erythrocytic stages. The sporozoite is accessible to antibody while in the skin and bloodstream, and therefore much effort has focused on sporozoite surface antigens, most famously the major surface antigen called circumsporozoite protein (CSP). During LS development, parasite encoded proteins, including CSP, can be the target of $CD4^+$ and $CD8^+$ T-cell-mediated effector mechanisms that confer protection in animals [reviewed in (1)]. However, few LS antigens have been identified until recently, owing to the relative inaccessibility and paucity of the LS parasite in vivo, and our inability to grow *Plasmodium falciparum* LS parasites *in vitro*. Technical advances now allow the enrichment of sparse intrahepatocytic parasites from abundant host material (2), as well as improved *in vitro* LS culture (3), including the axenic (or host cell-free) growth and differentiation of LS parasites (4).

Proteins expressed by pre-erythrocytic parasites have strong evidence and appeal as vaccine antigens. First, the mosquito transmission event represents a bottleneck in the parasite lifecycle. Only a few dozen or hundred sporozoites are transmitted to the mammalian host (5), and not all of these reach the liver (6). The low parasite numbers increase the likelihood for complete elimination by vaccines, and reduce the likelihood that resistant mutants will emerge, in contrast to erythrocytic stage parasites that can rapidly multiply to number in the trillions in a single individual. Second, sterile immunity against LS parasites is achievable. Protective immunity was demonstrated in rodents in 1967 by Ruth Nussenzweig using radiation-attenuated sporozoites as vaccines (7), and shortly thereafter was achieved in humans (8). Scientists are now developing methods to manufacture irradiated sporozoites as a vaccine product (9), which at present requires that parasites be dissected from infected mosquitos. More recently, genetic attenuation has been used to modify rodent parasites that arrest during LS development and induce protection (10,11). Genetically-attenuated *P. falciparum* parasites are currently being prepared to test this vaccine concept in human trials (12,13).

Third, subunit pre-erythrocytic antigen vaccines have already elicited partial protection in human volunteers. A vaccine targeting CSP, expressed as a recombinant protein fused to hepatitis B surface antigen to form a viral-like particle and referred to as RTS,S, is already in late-stage clinical trials. In phase IIb trials in Mozambique, RTS,S formulated with the adjuvant AS02 had an adjusted vaccine efficacy against clinical malaria of 35.5% in infants over 6 months of follow-up (14), and of 35% in older toddlers sustained up to 18 months (15). In Tanzania, RTS,S/AS02 co-administered with standard infant vaccines did not interfere with antibody responses to the latter, and maintained efficacy against clinical malaria (16). RTS,S formulated with the adjuvant AS01E achieved vaccine efficacy of 53% over 4.5–10.5 months of follow-up among 5- to 17-month-old children in Kenya and Tanzania (17), exceeding the efficacy observed with RTS,S/AS02 in Mozambique.

Importantly, while the long-term antibody response against CSP in vaccinated children in Mozambique waned, protection against clinical malaria was evident for up to 21 months (15). RTS,S also appeared to reduce pneumonia hospitalizations in Tanzanian infants (16), and severe adverse events in East African infants and toddlers (17), compared to control vaccinees. Notably, by the end of follow-up in the Mozambique studies, nearly 80% of

vaccinated children had experienced a parasitaemia (15). These trials suggest that RTS,S confers protection from clinical malaria and potentially other severe adverse events, but does this without completely preventing infection. The immune basis for RTS,S-induced protection has yet to be fully defined (17), although CD8 T cells do not appear to be involved (18). Further, the absence of sterile protection suggests significant scope to improve upon the efficacy of RTS,S.

The efficacy of RTS,S might be improved with more powerful adjuvants (17), or by the incorporation of additional protective antigens. In mice immunized with attenuated parasites, the CSP protein is an important but not exclusive target of protective immunity. For example, in CSP-transgenic mice that are tolerized to CSP antigen, protection afforded by irradiated *P. yoelii* sporozoites was reduced but not ablated (19). Mice immunized with irradiated *P. berghei* sporozoites were completely protected against transgenic *P. berghei* parasites expressing *P. falciparum* CSP, even in the absence of a functional response against the *P. falciparum* antigen (20). These elegant studies suggest that additional LS antigens can mediate protection, and offer models to delineate the relative contributions of CSP and novel antigens to protective immunity.

Another vaccine designed to induce protective immunity against pre-erythrocytic parasites, a composite antigen comprised of multiple epitopes fused to thrombospondin-related adhesion protein (ME-TRAP), has been tested in humans. Preliminary trials using a DNA-primed, viral-vectored boost immunization schedule in malaria naïve subjects produced a significantly delayed onset of blood stage parasitaemia (21). However, in Phase IIb trials in semi-immune adults, the vaccine was highly immunogenic but not protective in 372 Gambian men (22). Subsequently, 405 Kenyan children were immunized in a prime/boost system involving two viral vectors; these children generated moderate immune responses against the TRAP protein but also were not protected (23). Malaria exposure might have modulated the vaccine-induced immune response in the Gambian and Kenyan semi-immune populations. The studies emphasize that partially protective immunity achieved with a vaccine in malaria-naïve populations might not predict outcomes in malaria-experienced individuals.

MECHANISMS OF PRE-ERYTHROCYTIC IMMUNITY

While the intrahepatic immune response against *P. falciparum* parasites is extremely challenging to study, the parallel process in the mouse model has been relatively well characterized. Like wild-type parasites, irradiated sporozoites invade hepatocytes. However, shortly after infection their development arrests at the uninucleate trophozoite stage (24). The infected hepatocyte is a primary target of protective immunity in the murine irradiated sporozoite model (25), and antigens expressed during the early LS, therefore, represent potential targets of protective immunity. The extended presence of arrested parasites in the MHC-I-expressing hepatocyte may render parasite antigens more accessible to cell-mediated immune responses (26), or attenuation may disrupt parasite mechanisms for subverting host acquisition of protective LS immunity.

The irradiated sporozoite model of immunity has allowed the cells mediating protection in mice to be identified. Protection involves several mediators and can vary between models. Depletion studies show that $CD8^+$ T cells play a central role in clearing LS infections in some animal models (27), and CSP-specific $CD8^+$ T cells above a specific level correlate with protection in some models (28). Injection of IFN-γ alone can inhibit LS parasite growth (29), suggesting that this cytokine is a key effector molecule involved in protection. However, protection against irradiated parasites can also be achieved in the absence of $CD8^+$ T cells (30). Additionally, $CD8^+$ T cell-mediated protection is largely abolished when $CD4^+$ T cells are depleted (31), demonstrating that $CD8^+$ T cells are not sufficient to confer immunity in some models. The genetic background of both parasite and mouse impacts the nature of the protective response. For example, anti-IFN-γ antibodies ablate protection in the *P. berghei* system, but may have a limited effect in the *P. yoelii* system (32,33). In addition to cellular responses, antibody responses such as those against CSP can clear sporozoites before hepatocyte invasion and contribute to protection (34).

Although irradiated sporozoites confer full protection in humans, and RTS,S achieves partial protection, we nevertheless understand little about protective immunity induced by either vaccine strategy in humans. Irradiated sporozoite vaccines do induce cellular responses in humans (35), but CSP-specific $CD8^+$ T cell responses are largely absent from individuals immunized with RTS,S (18), implying that this important effector of protection in mice may not be relevant to the type of protection induced by RTS,S in humans. In Gambian adults immunized with RTS,S, CSP peptide-specific IFN-γ-secreting T cells measured in a cultured ELISpot assay were significantly associated with vaccine-induced protection (36). By contrast, antibodies and proliferative responses to CSP antigens did not correlate with protection. Our limited ability to identify robust correlates of protection in humans may indicate that diverse mechanisms, or combinations of mechanisms, can confer resistance and these might vary between protected individuals. Without clear and consistent correlates of protection, the selection of antigens for next generation vaccines is problematic, and must rely on parallel lines of evidence and inconclusive criteria.

A NEW UNIVERSE OF LS ANTIGENS

High-throughput approaches to define LS gene and protein expression profiles have increased our understanding of parasite biology, and provided a more comprehensive list from which to select next-generation vaccine candidates. Since 2002, the genomes of six key *Plasmodium* species have been published. Genome data for three species infectious to humans (37–39) and three rodent model species (40,41) have been completed. For most of these species, data comparing the blood stage transcriptomes and proteomes are also available [reviewed in (42)]. Progress on expression profiles has been slower with LS parasites, which have been technically difficult to isolate in sufficient quantities for any of the *Plasmodium* species.

The pace of genome-scale LS studies is accelerating as parasite manipulation and genomic technologies improve. The development of axenic methods for cultivation of *Plasmodium* sporozoites into early LS-like parasites (4) allowed the partial definition of the *P. yoelii* 24 h LS transcriptome (43). Axenically grown *P. yoelii* parasites yielded expressed sequence tags

(ESTs) representing 652 unique transcripts in the first study to detail at a genome-scale the transcriptional changes associated with the switch from sporozoite to LS. Many hallmarks of this transformation were detected; for example, the axenic parasite expresses A-type ribosomal RNAs (4), which are characteristic of development toward the blood stages. This suggests that transcriptional changes detected in this type of parasite are biologically relevant. Laser capture microdissection has also been used to isolate infected hepatocytes from host material (44). *Plasmodium yoelii* LS schizonts were micro-dissected from mouse liver, and the resulting ESTs were sequenced, revealing 623 *P. yoelii* genes expressed at 40 h of LS development.

Infection-associated transcriptional changes that occur in salivary gland sporozoites of *P. falciparum* after exposure to host cells have recently been profiled (45). Sporozoites co-cultured for 1 h with human hepatocytes in tissue culture increased their transcription of 532 genes, compared to nonexposed salivary gland sporozoites. Transcription of 21 genes was confirmed by quantitative PCR, and the expression of four encoded proteins was confirmed in sporozoites or infected hepatocytes. Two sporozoite proteins (PFD0425w and PF08_0005) localized in a pattern similar to CSP, suggesting display on the sporozoite surface. Antibodies against both proteins inhibited sporozoite invasion of hepatocytes, although to a lesser degree than anti-CSP antibodies. Because antibodies against PFD0425w can be detected in irradiated-sporozoite immunized humans (46), it will be valuable to assess whether antisera raised against PFD0425w and CSP in combination may be additive or synergistic for inhibiting sporozoite invasion. The other two proteins, PFL0065w and PFB0105c, were detected in the LS parasite, but not in the sporozoite. Both proteins were immunolocalized at the periphery of the early LS parasite, similar to the pattern seen with CSP. PFB0105c contains a putative PEXEL domain, and PFL0065w contains a predicted signal peptide, consistent with their localization at or near the PVM.

Using flow cytometry to sort GFP-expressing *P. yoelii* LS parasites, Tarun *et al.* (47) were able to obtain sufficient parasite material from infected rodent livers for DNA microarrays and for proteomics studies. Approximately 1100 genes were found to be differentially expressed at each of three mid- to late-LS timepoints, compared to blood stage parasites and to sporozoites. In parallel, 712 proteins of *P. yoelii* LS parasites were identified by mass spectrometry, including a so-called secretome of 93 proteins with putative signal peptides and 76 with predicted transmembrane domains. Secreted proteins will presumably be more accessible to the host proteasome, and it will be useful to assess whether these are more likely to elicit cellular immune responses or to confer protection as vaccine immunogens. The vaccine antigen CSP, for example, contains PEXEL/VTS motifs that mediate its export across the parasitophorous vacuole and into the hepatocyte cytoplasm (48).

Functional genomics studies of LS development have thus far profiled the transcriptome of *P. falciparum* at the hepatocyte invasion timepoint, and the transcriptome and proteome of the mid-to-late LS *P. yoelii* parasite. Expression profiles from early LS *P. yoelii*, and from early and late LS *P. falciparum*, remain to be completed. Because radiation-attenuated parasites arrest during early LS development (24), antigens expressed at this stage might be particularly valuable for subunit vaccine development. Proteomic analyses of *P. falciparum* in both the early and late LS await technical advances. Similar to the progress made with

GFP-expressing *P. yoelii*-infected hepatocytes, methods for enrichment of *P. falciparum* LS parasites will be useful for expression profiling of these key vaccine targets.

PRIORITIZING NEXT GENERATION VACCINE ANTIGENS

In the absence of a single protective immune effector to guide the selection of pre-erythrocytic vaccine antigens, the candidates can be comparatively assessed in several models of immunity to determine which consistently display the features that might predict protective efficacy. Using CSP as a benchmark, an ideal candidate antigen would be present in the LS parasite, effective as an immunogen to protect mice, a target of immune effectors especially $CD8^+$ T cells in rodents protected by attenuated parasite vaccines, and a target of immune effectors in humans protected by attenuated parasite vaccines or by naturally occurring exposure (Figure 1). The antigens that best meet these criteria could then be assessed as immunogens in human challenge studies, to confirm which should proceed to field testing and efficacy trials. Candidates that were synergistic with CSP immunogens for inducing protection in animals might be of particular interest, as a strategy to enhance the efficacy of RTS,S by incorporating additional antigens.

Mouse immunization studies: predicting vaccine efficacy in humans

DNA vaccinations have been used to screen panels of parasite genes as immunogens for protection in mouse models. In a survey of 19 *P. yoelii* genes from a sporozoite cDNA library, only CSP and the novel antigen PY01316 prepared as single-component DNA vaccines could protect Balb/c mice from subsequent sporozoite challenge (49). Of note, CSP elicited protective immunity when delivered by intramuscular inoculation but not by gene gun, whereas PY01316 elicited protective immunity when delivered by gene gun but not by intramuscular inoculation. Different pre-erythrocytic antigens might be targeted by different effector mechanisms involved in protection, and therefore formulations or routes of delivery required to confer protection may vary depending on the antigen.

Immunization studies can assess candidate immunogens for their efficacy against different parasite species and across different mouse genetic backgrounds. The partial protection afforded by CSP in clinical trials is mirrored in the partial protection of CSP as a DNA, protein, or viral vectored immunogen in multiple immunogenetic backgrounds of mice [reviewed in (1)]. In mouse systems, this makes CSP a useful benchmark for comparative studies, and allows immunogens to be combined with CSP to determine which may increase the degree of protection achieved with CSP alone. Notably, several antigens that conferred protection in mice failed to elicit protective immunity in humans when the *P. falciparum* orthologue was used, emphasizing that mouse studies are imperfect predictors for successful human vaccines, and should be interpreted with caution (21,50).

Attenuated sporozoite model: defining antigens associated with sterile immunity

The immune mechanisms conferring protection in mice after vaccination with attenuated parasites have been described in some detail (see above). In several of these models, IFN-γ-expressing $CD8^+$ T cells play a key (28) but not always exclusive role in protection. CSP-specific antibodies, $CD4^+$ T cells, and $CD8^+$ T cells can each confer protection when

passively transferred to naïve mice (34,51,52). Thus, pre-erythrocytic antigens can be comparatively assessed for reactivity with cellular and humoral responses in protected mice. Such studies can use radiation-attenuated sporozoites, or genetically attenuated sporozoites, as immunogens. Different genetically attenuated parasite vaccines were recently observed to confer different degrees and durations of protection (53), although other studies suggest similar levels of protection induced by radiation attenuated and different genetically attenuated parasite vaccines (54). A panel of whole parasite vaccines that varied in their efficacy might enable a more refined interrogation of protective immunity, and a better definition of the antigens specifically related to long-term protection.

Importantly, parasite attenuation is not required to induce protection. Live wild-type sporozoites can induce sterile protection directed against LS parasites when mice are inoculated and treated with chloroquine (55) or primaquine (56), a strategy termed infection-treatment vaccination. Infection-treatment vaccination with blood-stage parasites and chloroquine also confers protection, with immune responses directed to blood stage but also sporozoite and LS parasites (57). These models of protection that use wild-type parasites as vaccines might be useful for revealing novel mechanisms and targets of protection.

Our understanding of protective immunity in humans vaccinated with attenuated sporozoites is more rudimentary. CSP-specific antibody and cellular responses develop in irradiated sporozoite-immunized individuals (35), and their T cells proliferate in response to LSA-1 peptides (58). Based on knowledge gained from mouse models, LS antigens targeted by $CD8^+$-IFN-γ responses would be of great interest as vaccine candidates. However, CSP-specific immunity confers protection in RTS,S-vaccinated individuals in the absence of detectable $CD8^+$ T cell responses (18). $CD4^+$ T cell IFN-γ responses to LS proteins and functional antibodies to sporozoite surface antigens that develop after vaccination with attenuated sporozoites can be considered criteria for antigen prioritization in addition to $CD8^+$ T cell IFN-γ or polycytokine responses.

Naturally acquired immunity: assessing the effect of malaria on malaria vaccines

The ME-TRAP vaccination studies suggest that natural malaria exposure can subvert the development of partially protective responses after subunit vaccination. In naturally exposed Kenyans, memory T-cell IFN-γ responses against TRAP correlated with reduced malaria incidence (59), but these responses may be subverted during malaria episodes (60). Therefore, candidate antigens should be assessed by measuring antigen-specific responses in naturally exposed individuals and associating those responses with protection. Studies of the LS-specific protein LSA-1 have revealed the complexity of the immune response directed against LS parasites in naturally exposed humans [reviewed in (61)], and suggest that naturally acquired immunity to LS parasites is involved in protection. LSA-1-specific IFN-γ responses have been associated with an increase in time to next infection and a lower overall parasitaemia in children in Gabon (62). IL-10 responses to recombinant LSA-1 protein fragments were predictive of increased time to next infection and reduced parasite density and frequency in a treatment-reinfection study in adolescent and adult Kenyan males (63). Antibody responses to LSA-1 have been associated with a reduction in the risk of clinical malaria and a reduction in cumulative incidence of malaria in Kenyan children (64).

Collectively, these studies show that wild-type LS parasites are immunogenic in naturally exposed humans, and that immune responses to LS parasites correlate with protection in endemic populations including children. Similar studies should compare novel LS antigens to CSP and LSA-1 as targets of immune responses that correlate with protection. More evidence is needed to know whether pre-erythrocytic antigens identified by immunocorrelation studies in naturally exposed individuals are more likely confer protection when used as vaccines. Such studies may also give insights into which vaccine-specific immune responses occur naturally, and therefore will be boosted by exposure to the parasite after immunization.

CONCLUSION

In humans, complete protection against malaria can be induced by immunization with irradiated sporozoites, and partial protection from clinical disease occurs after vaccination with the CSP-based subunit vaccine RTS,S. Efforts are underway to develop processes needed to manufacture and vial irradiated sporozoites as a deliverable vaccine, and the initial trials using this product are imminent (9). Ultimately, a subunit vaccine would be preferable owing to ease of manufacture and delivery. The selection of novel pre-erythrocytic antigens for subunit vaccines is hindered because we do not understand protective immunity in humans. CSP is expressed on the surface of sporozoites where it is a target of antibody, and is subsequently carried into the hepatocyte by the LS parasite where it can be a target of $CD4^+$ and $CD8^+$ T cell responses. CSP can therefore serve as a model for studies of novel pre-erythrocytic antigens, and provides a useful benchmark for comparisons of immunogenicity and antigenicity. Genome-based studies have defined in part the universe of pre-erythrocytic proteins that can be considered for next generation vaccines; the transcriptome and proteome of early LS parasites remains to be determined. These novel LS proteins, along with sporozoite antigens, should be carefully assessed in our existing models of immunity –whole organism vaccination, subunit vaccination, and naturally acquired protection – to determine which antigens have the best combination of features that suggest likely efficacy as vaccines in humans. The availability of a human challenge model allows us to assess a limited number of immunogens in a highly controlled setting, before recommending those candidates that should proceed to larger field trials. Recent advances portend further progress in the development of pre-erythrocytic malaria vaccines, building on earlier successes such as attenuated sporozoite vaccines and RTS,S.

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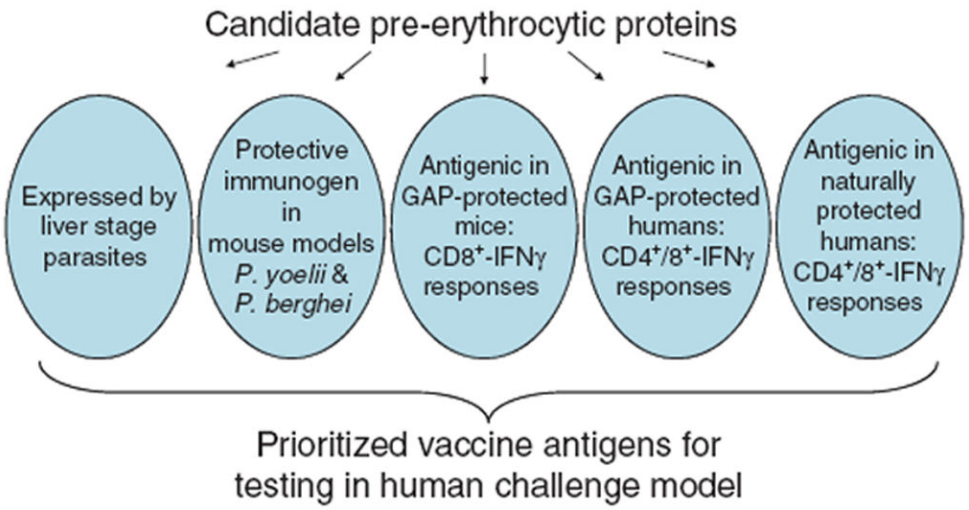

Figure 1.

Potential criteria for selection of candidate antigens for pre-erythrocytic malaria vaccines. Hundreds of candidates have already been identified in functional genomics studies of pre-erythrocytic parasites, and future studies will likely expand this list, emphasizing the need for a systematic process of assessment and prioritization. Proteins expressed by liver stage parasites can be compared by several criteria that may be relevant to their potential role as human vaccines, such as their antigenicity in protected animals and humans, and their efficacy as vaccines in animal models of malaria. The candidates that meet most or all these criteria can be validated in human challenge trials, in which malaria-naïve individuals vaccinated with candidate immunogens receive sporozoite challenge by infected mosquito bite. Immunogens that prevent infection (i.e. appearance of blood stage parasitaemia) can be transitioned to field testing and efficacy trials in endemic areas. GAP, genetically attenuated parasite.