Pre-erythrocytic stage malaria vaccines: time for a change in path

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Vaccines against the pre-erythrocytic stages of malaria hold the greatest promise as an effective intervention tool against malaria, as shown by immunization with radiation-attenuated sporozoites over four decades ago. To date, however, the development of subunit vaccines, while generating high expectations and investment, has not lived up at all to the promise. This path has been characterized by insufficient research into both identification of key defense mechanisms in humans and the discovery of better antigens, focusing rather on a technological race of how to present mainly a single antigen. The lack of success has also led, perhaps from desperation, to a revival of the live attenuated sporozoite approach, handicapped, however, by major bottlenecks in production, safety, and regulatory issues. It should now be clear that the field can no longer continue to succeed in mice and fail in the clinic. We advocate here in favor of a third option, relying on an understanding of the basis of attenuated sporozoite immunity in humans, to provide leads to the discovery of critical immunogens and the use of models with validated relevance to the human situation in order to rationalize and renew the promise of pre-erythrocytic subunit vaccines.

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Introduction

There are more than a hundred species of malaria parasites, four naturally infect humans, which usually induce species-specific immunity [1,2]. The malaria parasites undergo three different modes or stages of reproduction: sexual in the mosquito host to produce infective sporozoites and two distinct asexual replications, first, one in the hepatocytes of the liver and, thereafter, another in the red blood cells. At each stage the parasites dramatically change their gene expression. This translates into major differences in morphology, metabolism, and antigen content with the latter leading to the generation of stage-specific immunity [3•]. The pre-erythrocytic stage vaccines (PEV) include antigens from the sporozoite and liver stages.

The promise of PEV stems from the remarkable feature that they can induce a state of strong, sterile immunity both in humans and in animal models. This is a very rare and striking phenomenon, as most parasites usually induce suboptimal immune responses that merely tend to control parasite loads without elimination, resulting in chronic carriage.

For the above reason, PEV have generated very high expectations, attracting strong-willed intellects with ambitious personalities who are able to successfully advocate for large programs. As the primary target population for PEV are individual travellers (tourists, businessmen, and armed forces personnel) there is a potential multi-billion dollar market with PEV attracting a high proportion of vaccine research funding. Nevertheless, saving the lives of children from endemic areas is frequently put forward to justify the large investments. At the other extreme end of the vaccine-funding scale stand the altruistic gamete vaccines aimed at protecting poor endemic communities by blocking transmission [4].

Another striking feature of PEV is that the scientific paradigms of protection have dramatically changed over time and so have the development strategies. The immune mechanisms assumed to be important have bounced from antibodies altering the sporozoite surface, blocking mobility or inhibiting their invasion to the induction of cells cytolytic to the infected hepatocyte, or cytokines (IFNγ), or free radicals inhibiting intra-hepatic parasite development [3•]. Accordingly, the technological tools for presentation of PEV antigens have been remarkably diverse covering a wide range from disincarnated DNA to the whole living but attenuated parasite.

PEV research, which has been mainly conducted using species of rodent malaria parasites with mice as hosts has led to, with one exception, the design of vaccines that proved very successful at protecting mice but so far has failed to achieve the same success in humans. As several in-depth reviews have recently covered the state of PEV technological development [5,6•], we will critically examine here the two radically opposite research approaches for PEV, subunit vaccines and whole attenuated parasite

vaccines, with the aim to suggest actions that could move the field forward.

The foundation

Serendipity is frequently an occurrence somewhere along the path of major discoveries and an accident, a carton of infected mosquitoes left under UV light, was the initial event that eventually led to 40 years of research to find an effective malaria vaccine. The UV-'inactivated' *P. gallinaceum* sporozoites were no longer infective for chickens, yet induced strong protection against subsequent challenges of virulent sporozoites [7,8]. Substituting gamma radiation for UV inactivation and mice and humans in place of chickens, this initial observation was extended to rodent and human malaria parasites [9–15], providing a proof of principle that launched the current vaccine races.

The subsequent research path taken toward development of an effective PEV stands in sharp contrast, though, to the elegant simplicity and sound reasoning that generated the initial discovery of solid protection afforded by radiation-attenuated sporozoites (RAS) [10,11,15•]. At that time it was pragmatically believed, perhaps rightly so, that development of an RAS-based vaccine was not practical. Subsequently, with the identification of the gene for the circumsporozoite protein (CSP) [16], emphasis of the next 27 years of PEV research has centered on serial application of the latest available technologies toward this goal.

A remarkable showcase of technologies: subunit vaccines

Indeed efforts have concentrated far more on varying methods of presenting the first PE antigen identified than at identifying the vaccine potential of the remaining 2000 pre-erythrocytic antigens, or at understanding the physiology and the biology. The CSP has been, and still is, the object of numerous trials based on a variety of peptides, recombinant proteins, modified virus vectors, plasmids, and a large diversity of adjuvants and immunization regimens. The vast majority has failed to induce any protection though succeeding at adsorbing large amounts of money.

The biggest investments and expectations have come recently from RTS,S, a particulate formulation of recombinant CSP fused with the hepatitis B surface antigen. RTS,S in combination with the saponin-based adjuvant AS02A is, to date, one of the rare subunit vaccine candidates to have shown some level of efficacy, though quite modest, in clinical trials. Initial Phase Ib clinical trials were very promising with six of seven volunteers protected from challenges with parasites expressing the same CSP allele as that used to prepare the vaccine [17•]; only one of the original six was protected when re-challenged six months later [18]. However, a more recent trial comparing AS02 with AS01 led to protection in two out of eight, retrospectively raising doubts about the reproducibility of the initial result. In total, initial protection with RTS,S immunization of volunteers given homologous challenges has been about 40% [19], though appropriate control groups were apparently never included.

In field studies in the Gambia [20] and in Mozambique [21,22], where natural challenges result from exposure to many different CSP alleles, RTS,S in these trials achieved a few months delay in the occurrence of initial malaria attacks in 30% of the vaccinated population. In general the immunity engendered by RTS,S appears to wane quickly over time without significant reduction in the total number of malaria attacks over the study period. Incidence rates were 62 and 67% or 87 and 94% in vaccinees and controls, in the Gambia and Mozambique, respectively, where substantial levels of transmission were taking place.

The apparent occurrence of a 48% reduction in severe malaria cases (3% versus 7% incidence in vaccinees versus controls) has undoubtedly contributed to expanding the visibility of RTS,S [6•,21,23]. This result was unexpected, as the vaccine does not target erythrocytic stage parasites, which are responsible for the clinical manifestations of severe malaria. Indeed, there was no reduction in parasite densities (higher densities are positively associated with pathology) among cases occurring in vaccinees as compared with controls, whereas this would be expected for an effect on erythrocytic stage disease. Above all, the trial was not powered to explore an infrequent event such as severe malaria. Hence, this result may have received an inordinate degree of attention and should have been the object of additional investigations with stringent case definition [24•].

The story of the Spf66 vaccine, which a decade ago also achieved the same promise of a 30% or greater reduction in morbidity, had demonstrated how costly and time-consuming it can be to conduct trials when initial results based on insufficiently designed studies created big expectations. Over the course of 10 trials, requiring eight years to implement, SPf66 efficacy declined from over 60% to essentially nil [25,26].

What is the true value of RTS,S as a deployable 'anti-disease' vaccine? Despite questions about an overall modest level of efficacy and short duration of activity [24•], the MVI/GSK collaboration appears to have decided to scale up the RTS,S adventure to a large multi-center Phase III trial involving up to 16 000 infants; a step closer to obtaining licensure. There has been a call for the United Kingdom and other northern governments to underwrite the skyrocketing costs for the future deployment of this vaccine, underscoring a recent trend where policy issues are becoming as decisively important as scientific ones. This might be thought surprising given

the fact that there are effective drugs that can achieve this level of efficacy (or greater) at much less cost.

Protagonists argue that the governments in need of this vaccine are not financially able to cover purchase costs and industry needs to be assured of sales to move forward despite a modest impact [27]. But it has also been cogently argued that this is not good policy and will only serve to institutionalize an inferior vaccine, lessen political commitment to malaria vaccines, and inhibit the development of vaccines capable of giving better results.[a]

Other, mainly technology driven, approaches to PEV have relied on the CSP or TRAP (a sporozoite invasion ligand) genes, or both, or synthetic multi-epitope genes, recombined either in plasmid DNA or in viral vectors such as vaccinia (MVA) or fowlpox (FP9), given either alone or in a series of more or less complex 'prime-boost' regimens of one followed by the other or even by the recombinant protein. Several combinations were even employed, apparently, with the questionable rationale that combining two inefficient vaccines might produce an efficient one. With the exception of one trial, which indicated a slight delay in patent blood infection in non-immune volunteers [28], this and remaining Phase I and Phase II trials have failed to provide any evidence of preventing infection or clinical disease [29–32]. The next popular technological step ahead relies on the same antigens delivered by yet another platform, adenovirus [33–35], which, as always, is assumed to be the path to the Holy Grail.

Those waiting in endemic regions should not be misled by the size of the investments, the press announcements, or the array of novel technologies, especially, given the modest achievements for PEV today.

The live attenuated vaccine alternative: reality or strategy of despair?

The concept of live attenuated whole parasites as a malaria vaccine is undergoing a resurgence in popularity after more than 30 years of dormancy. It is an old-fashioned approach that recently acquired a new twist. In parallel to random mutation by irradiation, the newest incarnation achieves attenuation through modern genetic prestige by knocking out genes to curtail intra-hepatic development [36•,37,38]. Application of this state of the art technology to create genetically attenuated sporozoites (GAS) may make the concept smart and fashionable but does not alter the challenges in developing and delivering this live vaccine.

Nevertheless, the attenuated vaccine approach is being re-invigorated in a disheartening landscape. Its characteristics are directly opposite to those of the subunit PEV approach in almost every aspect including efficacy, production, delivery, safety, and regulatory issues. The expected high efficacy of the RAS vaccine stems from very convincing, but limited, data involving less than two dozen human subjects over a span of 25 years [39]. There are, though, even much less data about its duration. Results with the genetically attenuated sporozoite, by contrast, stem solely as yet from *P. berghei* knockout experiments in rodents.

The bottlenecks and barriers facing development of RAS or GAS vaccines are seemingly unlimited when closely examined. First, in order to produce stable GAS vaccines, several virulence genes need to be identified and knocked out since single gene KOs, such as *uis3*, have shown partial attenuation with parasites capable of breaking through to produce blood infections [36•]. In the absence of a suitable animal model for *P. falciparum*, this implies that human volunteers will be needed to assess sporozoites attenuation for each individual candidate gene one by one (and thereafter vaccine efficacy on virulent challenge), even before combining several KOs in a single clone. It, of course, cannot be assumed that genes of crucial importance as targets in rodent species will have the same relevance in *P. falciparum*.

Secondly, administration and delivery of these RAS or GAS live vaccines, which currently require cryopreservation and transportation at ultra-low temperature in liquid nitrogen, will also cause innumerable logistical headaches, as it is already difficult to provide vaccines that merely require a +4 °C cold chain. Likewise, a third challenge is the route of administration since immunity is, in principle, dependant on the intra-hepatic invasion by a live parasite and transformation into an arrested liver stage trophozoite [3•,40,41]. Whether the live parasite can also be efficiently cryopreserved and immunity be also induced by routes of administration other than IV (rodents) or mosquito bite (humans) is unknown. The only known effective regimen for humans requires 10–12 immunizations (i.e. requiring about a year), each represented by exposure to a few hundred feeding RAS-infected mosquitoes [39]. Whether other immunization regimens can achieve the same result remains to be investigated in clinical trials.

Perhaps the greatest barriers pertain to safety and regulatory issues [42]. The production of sporozoites, which can take place only *in vivo* in the mosquito host, raises technical and reproducibility difficulties. Above all, successful infection of mosquitoes requires human red blood cells and serum, which by necessity come from many different donors, and which, therefore, multiply accordingly by touchy quality control steps. The risk of carrying known pathogens besides unknown ones that may emerge is well known by blood banks and is not negligible. This risk,

[a] (www.economics.ox.ac.uk/members/andrew.farlow/FarlowMalaria.pdf).

which is acceptable for therapeutic reasons in a single individual, will probably be unacceptable for prophylactic reasons when thousands of healthy individuals could receive vaccine batches each derived from one blood donor. At a time of the emergence of numerous blood-borne diseases when the use of fetal calf serum, which in addition can be sterilized by heat or irradiation, is being excluded from vaccine production, a regulatory authority may have some difficulty accepting a vaccine manufactured using human red blood cells, which cannot be sterilized. Nevertheless, despite the many challenges, eight-figure funding has been recently committed to produce RAS and GAS vaccines in semi-industrial fashion.

The attention and research efforts being placed into attenuated PEV may, to some extent, be in reaction to what is perceived as failures of the subunit vaccine approach. It should be noted, though, that the latter has to date concentrated mostly on only two sporozoite molecules (CSP and TRAP) out of several thousand expressed by pre-erythrocytic parasites. Hence, there ought to be little reason for desperation at this point, as most antigens have yet to be investigated.

The consequences of original sin

The reasons why vaccines work better in rodent models and conversely often fail in humans may best be understood in the context of the immunological consequences of the molecular fine tuning that molds parasites evolving within their natural hosts. Malaria vaccine discovery and development requires a reliance on some initial method(s) to screen candidates [43]. The consequences of the screen(s) employed to identify a vaccine candidate have seldom been critically addressed. Depending on reliability and relevance the initial screen(s) could constitute the 'original sin' in malaria vaccine development, from which the consequences may be, thereafter, carried out over time through an unlimited number of permutations.

Plasmodium species under natural circumstances are strictly fitted to mostly ONE given host or very closely related group of hosts [1]. If introduced in an abnormal host, they either die off after relatively short courses of infection, create a fulminant infection or fail to establish productive infections. Conversely in their normal host where they are 'adapted', they do not necessarily kill their hosts (or rarely at a high rate), and, conversely, all are not killed by their host, with the result being chronic, long lasting, low grade parasitemia, which is the rule. Examples are *P. falciparum* or *P. vivax* in humans, the rodent malaria parasites *P. berghei* or *P. yoelii* in African rodents, *Grammomys surdaster* and *Thamnomys rutilans* or the numerous simian malaria species in the macaque monkeys of Asia.

Obviously, this equilibrium has a molecular basis. The 'adaptation' of a parasite to its host through co-evolution over thousands or millions of years, during which they undergo by chance a huge number of random mutations leads to the selection of parasite molecules that have by necessity two main characteristics: (a) They do not induce too much pathology and (b) they do not trigger defenses that are too effective. Parasite mutants that do not satisfy these two criteria have disappeared either by killing their host or being killed by it without efficient completion of the life cycle. However, almost all experimental malaria models employed to pre-clinically select potential PEV candidates are not normal host–parasite combinations; most often being one of the rodent parasite species in the mouse, an unnatural host for these parasites [44].

In an abnormal host this molecular fitness is lost or not highly tuned. For vaccine development, this situation has important consequences as the molecular mismatch implies that a larger number of molecules can induce protection either because they are abnormally expressed in the hosts cell membranes or they are more immunogenic than in the normal host, or because they induce more effective immune responses, or because the immune responses are directed to epitopes that interfere with parasite functioning, which are different from those able to be recognized by the normal host. Therefore, it should not be so surprising that the same molecules may more often fail than succeed when vaccinating humans.

The same holds true for models of protection induced by RAS. When comparing the natural host, a tree rat, with laboratory mice, protection appears to be inversely correlated with the susceptibility of the host to sporozoite infection. Results indicate that the protection is far easier to induce in the experimental than in the natural host–parasite combination [45,44]. For example, a single immunization with 1000 *P. berghei*-irradiated sporozoites can induce protection in BALB/c mice, but three immunizations of 100 000 RAS failed to protect in tree rats [46,47]. In humans 12–14 immunizations by exposure to hundreds of irradiated mosquitoes are necessary for protection [39].

A tale of mice, men, and wrong assumptions

Malaria vaccine development has, essentially, led to the design of several vaccines highly effective in mice and highly ineffective in humans. Indeed, it has been left, mostly, to the immune system of the mouse to choose the main horses to ride in this vaccine race. It was monoclonal antibodies from Balb/c mice that originally selected among a few thousand proteins the CS protein as the immunodominant antigen of sporozoite [48], which, while true in mice [49], has proven to be the opposite case in humans [50–52]. This fact was soon recognized, but rather than turning to efforts to discover better vaccine candidates, an interminable search was undertaken for magical adjuvants and vehicles able to turn a poor immunogen into a good one when given to humans. This moved the

research focus from a scientific perspective into the realm of technological issues and initiated two decades of clinical trials with a large number of delivery platforms, incurring corresponding high costs.

Inducement of high levels of cytotoxic lymphocytes (CTLs) has always been assumed to be an essential immune response necessary for PEV [53–55], though MHC Class I molecules, which are expressed at basal level on mouse hepatocytes, have not been shown to be present on *P. falciparum*-infected human hepatocytes. The prime-boost regimens employed by Hill and colleagues that successfully increased by 20–50-fold CTL activity in humans would seem to convincingly demonstrate that high-CTL activity, at least against CSP and TRAP, is not sufficient to provide protection in humans [29,31,56]. This result, which incidentally is in full agreement with data obtained with RTS,S, should have lead to the questioning of current assumptions about defense mechanisms against liver stages (LS) based on work in rodent malaria models.

The adjuvant AS02A was selected in mice among a large range of novel adjuvant combinations as the one best capable of inducing CTL activity [57]. When used in humans with RTS,S this adjuvant induced high CSP antibody titers, variable IFN-γ activity but no detectable CTLs [58]. This blurring of the initial rationale possibly makes it understandable why little effort was made later to understand the basis for the modicum of protection achieved with RTS,S.

A significant change in concept of PE immunity occurred when it was realized that solid protection was related to the capacity of an irradiated sporozoite to transform into LS and become arrested at some point during development [59,40,60]. The so-called sporozoite vaccine is, in fact, liver stage dependent. Rather than leading researchers to identify and characterize novel LS antigens, as this paradigm shift should have, the focus of development simply shifted from mechanisms targeting sporozoite invasion to those targeting intra-hepatic parasite development but retaining CSP and TRAP as the chief target antigens [3•]. Research conducted in rodents led to uncovering an extremely wide array of immune effectors. Nearly all investigated were found effective in one or the other of the many combinations of rodent *Plasmodium* species and mouse strains, however, without being able to determine if any had some potential in altering *P. falciparum* infection in the human host.

Thus, mouse models have occupied a supreme position in the development of PEV, yet their relevance has been seldom addressed. Mice have been employed as immunogenicity models and the rodent species of *Plasmodium* employed as models for protection in non-natural host/parasite combinations. In fact, the existing models seem to have been employed more on the basis of their ready availability in laboratory settings than on demonstrated scientific relevance.

Time for a change in path

There is now sufficient clinical evidence that the time has come to question the current path of PEV development. The rational basis for undertaking large development programs has been small, given the little investment placed in appropriately based investigations aimed at understanding immune mechanisms that would rationally guide development.

Investigations in humans are indeed difficult and limited, yet they might be unavoidable as they supply information that cannot be gathered by alternate means. In the recent past, several opportunities could have been taken [44]. For instance, when a combination of RTS,S and TRAP proved ineffective in the clinic as compared with RTS,S alone this could have supplied clues for identifying key mechanisms of defense in humans [61]. The same holds true for several RTS,S trials comparing different adjuvants with different clinical outcomes [17], or of an improved analysis of the 30% individuals showing in recent trials a delay as compared with those who did not. The current surge of clinical trials offers an opportunity to retrospectively address the validity of the initial paradigms.

Immunity elicited by live attenuated sporozoites in humans is probably the best 'model' of protection to concentrate on both for (a) deciphering the key protective immune mechanisms and (b) identifying their target antigens. In this aspect, little investigation has been undertaken or supported, and now these should be encouraged. It can no longer be considered as lost time, as it has been over the past two decades, to spent efforts trying to understand key defense mechanisms and immune targets to produce an efficient vaccine. For instance, comparative experiments in humans and relevant non-human primates, looking at optimally irradiated sporozoite-induced protection in comparison with overirradiated sporozoites that induce high immune responses, but no protection [40], could rapidly provide major clues [62•].

Although there is an obvious need to investigate the value of other antigens as vaccine candidates, there has been surprisingly little effort in that direction. This has been partly for the lack of appropriate methods [63,3], but presently such challenges are less daunting [64]. One early systematic approach led to characterization of 29 pre-erythrocytic *P. falciparum* molecules antigenic in exposed individuals [63,65]. Following the characterization of the first, LSA1 [66], a screening strategy based on protective human immune responses was chosen to explore the remaining antigens. LSA3 was identified in this manner, by the differential screening of immune

responses from protected versus non-protected human volunteers immunized with RAS [62•]. LSA3 was found able to confer strain-independent protection against *P. falciparum* challenges in immunized chimpanzees and *Aotus* monkeys [62•,67]. Clinical trials have yet to be implemented for LSA3 to learn if protection will extend to humans. Nonetheless, it is LSA1, which tested negative in the human protective immune response screen and failed to induce protection in chimpanzees that has been taken into clinical trials [6•,68], underscoring again the apparent lack of sound rationales currently prevailing in PEV development.

In summary, recent clinical results of PEV that have been in development for 20 years have been much less than expected and not very promising. Modeling malaria vaccines in mice and their failings in the clinic has now become patent. The high cost and slow speed of testing each new hypothesis in the clinic is leading to an unmanageable situation. It does not seem to have yet been fully realized that it is not realistic to evaluate through clinical trials each of the antigens, the many delivery platforms and adjuvant combinations, particularly when pre-clinical selection was built on faulty premises. Clinical studies may look straightforward and reliable but are less so than may seem at first, as definitions of clinical malaria are difficult, particularly in Africa where the majority of children are harboring parasites [69]. In practice, they also require large investments over several years to deliver the desired clues.

There is a need to fund the upstream pre-clinical research that fuels sound clinical development. This requires spending a very modest proportion of current investments (0.5–1.0%) to achieve a limited number of crucial steps, which are (a) identifying the mechanisms of defense prevailing in humans or reliable surrogates of protection, (b) characterizing the LS antigens targeted by them, and (c) evaluating the relevance of existing models and define better ones. There is no escape from the reality that improved rationales are needed to drive further PEV development.

In other fields of infectious diseases, such as AIDS, researchers would bargain with the devil to have access to a situation such as RAS-induced protection in humans capable of unraveling key defense mechanisms and antigenic targets, while malariologists have largely disregarded its value! This is the only model for induced sterile immunity to malaria in humans and must not remain a missed research opportunity. Any perceived delays arising from the need to understand the essential defense mechanisms prevailing in humans have to be balanced by ethical and financial concerns associated with the rising number of clinical trials performed with formulations designed on the basis of models of unknown relevance. Although 'research' is not always a word favored by vaccine developers and funders, we call for a limited, well-focused research component to be applied to these issues that are key to the successful development of PEVs. There should be little debate on this in view of the modest investment needed and the fact that this research could go forward in parallel to, not in place of, currently planned clinical trials.

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