A review of human vaccine research and development: Malaria

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Abstract

The last several years have seen significant progress in the development of vaccines against malaria. Most recently, proof-of-concept of vaccine-induced protection from malaria infection and disease was demonstrated in African children. Pursued by various groups and on many fronts, several other candidate vaccines are in early clinical trials. Yet, despite the optimism and promise, an effective malaria vaccine is not yet available, in part because of the lack of understanding of the types of immune responses needed for protection, added to the difficulty of identifying, selecting and producing the appropriate protective antigens from a parasite with a genome of well over five thousand genes and to the frequent need to enhance the immunogenicity of purified antigens through the use of novel adjuvants or delivery systems. Insufficient clinical trial capacity and normative research functions such as local ethical committee reviews also contribute to slow down the development process. This article attempts to summarize the state of the art of malaria vaccine development.

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Contents

1. Introduction ....................................................................................................... 1568
2. Disease burden .................................................................................................... 1568
3. Parasitology ...................................................................................................... 1569
4. Vaccines .......................................................................................................... 1570
  4.1. General considerations ....................................................................................... 1570
  4.2. Pre-erythrocytic vaccines .................................................................................... 1570
      4.2.1. CSP vaccines ....................................................................................... 1570
      4.2.2. DNA vaccines and live recombinant vaccines ......................................................... 1571
  4.3. Asexual blood-stage vaccines ................................................................................. 1572
      4.3.1. MSP-1, MSP-2 and RESA combination ............................................................... 1572
      4.3.2. MSP-1 and AMA-1 ................................................................................. 1572
      4.3.3. MSP-3, GLURP and SERA .......................................................................... 1573
      4.3.4. Other antigens ...................................................................................... 1573
1. Introduction

Malaria is by far the world’s most important tropical parasitic disease, killing more children aged less than 5 years than any other disease, especially in Africa [1]. Malaria is caused by the protozoan parasite Plasmodium, which is transmitted by the bite of the female mosquito of any one of the 50 species of Anopheles mosquitoes, of which the best known is A. gambiae [2,3]. Four species of Plasmodium can cause human malaria: P. falciparum, responsible for the greatest number of deaths, P. vivax, which has the widest geographical distribution, P. ovale and P. malariae [4]. The life-cycle and parasite–host interaction of each Plasmodium species determines the severity and pathogenesis of clinical disease [5]. As recently as the early 1900s, human malaria was endemic across every continent except Antarctica. By the 1950s, control programs based on the use of insecticides led to its elimination from North America, Europe and Australia, but the disease still remains endemic in Africa, South-East Asia and the Western Pacific region, where more than 40% of the world’s population lives.

The battle to control malaria has been fought on several grounds, including efforts to implement improved diagnosis and prophylactic chemotherapy as well as integrated vector control through the use of insecticide-treated bednets and residual house spraying [6]. However, prevalence as well as resurgence of malaria continues to be evident worldwide, much of it due to drug-resistant parasites and insecticide-resistant vectors. New malaria drugs such as chemical derivates of artemisin are too expensive to be affordable by poor countries and still in limited supply [7]. Therefore, the development of a safe, effective and affordable malaria vaccine is a critical global public health priority [8].

Eliciting protective immunity to malaria appears to be achievable, as judged by the fact that natural immunity to the parasite progressively develops with frequent exposure [9,10] and that this immunity can be transmitted by passive transfer of antibodies [11]. As early as 1967, experimental immunization with irradiated sporozoites was shown to generate protective immunity [12]. Proof-of-concept demonstration that protection can indeed be induced through vaccination with a subunit vaccine was recently obtained in children [13,14]. Progress in developing a malaria vaccine has, however, remained slow [15]. This is in part due to the fact that the Plasmodium parasite has more than 5200 genes that could code for a protective antigen [16,17], making identification of candidate vaccine antigens a real quagmire, which is rendered even more complex by the fact that these antigens are differentially expressed during the life-cycle of the parasite and that many of the antigens display a high degree of variability. Moreover, the same antigens can be developed into different types of vaccines in a wide variety of ways. Thus, more than 50% of the approximately 75 candidate vaccines in active development today, are based on just three antigens that were cloned 20 years ago [18]: the circumsporozoite protein (CSP), the merozoite surface protein (MSP) and the apical membrane antigen 1 (AMA1) [19–21].

There are reasons to believe that an efficacious malaria vaccine may have to include multiple antigens and target multiple stages of the parasite life cycle. Combination approaches however meet with many challenges, including possible competition between antigens and increased cost and complexity. In addition, the development of a malaria vaccine is met with technical, logistical and financial obstacles, including the scientific, medical and ethical considerations to be taken into account when planning and conducting clinical trials and the fact that the market for a malaria vaccine is viewed as commercially nonattractive. However, in view of the emergence of drug-resistant strains of malarial parasites, the need for the development of safe and effective vaccines for the control of malaria has never been greater, particularly vaccines against the parasite blood stage that is the cause of the clinical symptoms and mortality [22,23].

This article will attempt to summarize the state of the art of P. falciparum malaria vaccine development.

2. Disease burden

It is estimated that the global number of clinical cases of malaria is about 350–500 million per year, with an estimated annual death toll of over 1.1 million deaths. Approximately 800,000 deaths occur among children under 5 years of age in Africa, especially in remote rural areas with poor access to health services. Most affected countries are Uganda, Tanzania, Malawi and Mozambique as well as Namibia, where more than 200 cases are reported per 1000 inhabitants per year [1]. In these countries, malaria is by far the leading cause of absenteesism and death, even with the advance of AIDS. By contrast, the small number of cases still found in Europe (about 50,000 cases in 1999 [24]) and even smaller number in the USA (∼1500 cases [25]) are mostly imported cases in travelers or military personnel.

Transmission of malaria is affected by climate and geography, and often coincides with the rainy season. Global warming and other climatic events such as “El Niño” also play their role in increasing the risk of disease, presumably because the associated weather disturbances influence vector
breeding sites. Quantitative leaps in malaria incidence coincident with ENSO (El Niño/Southern Oscillation) events have been recorded in South America, Africa, and in Pakistan and Sri Lanka in Asia [26]. Thus, the disease has now spread to highland areas of Africa. A change in risk of malaria also can be the unintended result of economic activity or agricultural policy that changes the use of land, such as the creation of dams, new irrigation schemes, commercial tree cropping and deforestation [27,28]. Urban malaria is increasing due to unplanned development around large cities, particularly in Africa and South Asia. Injection or transfusion of infected blood or use of contaminated needles and syringes (e.g. injecting drug users) may also transmit malaria.

The incubation period from infection to symptoms ranges from 7 days to 18 days but may be as long as 40 days with \( P. falciparum \). Symptoms associated with malaria include high fever, malaise, chills, profuse sweating, headache, cough, arthralgia and myalgia, often accompanied by nausea and vomiting and at times diarrhoea. These attacks may last up to 12 h and classically recur every 48 h in tertian malaria (\( P. vivax \), \( P. ovale \) or every 72 h in quaran-tan malaria (\( P. malariae \)), in synchrony with the release of merozoites from lysed red blood cells (RBC). Anemia and splenomegaly most often develop after some days. \( P. vivax \) malaria, also referred to as “benign tertian malaria”, is rarely fatal because parasitemia is low grade. The main problems are fever and anemia, occasionally with massive splenomegaly.

Severe disease cases, which occur more frequently with \( P. falciparum \) because of its ability to infect a greater percentage of erythrocytes and to cytoadhere to capillary walls [5], are characterized by acute renal failure with hypotension and shock, or cerebral malaria with unrousable coma and convulsions, or pulmonary edema. These occur most commonly in populations that have not acquired immunity to malaria, such as young children and travelers [29]. Case fatality rates among untreated children and non-immune adult ages can reach 10–40% or higher. Fatally afflicted children often die less than 72 h after developing symptoms. In those children who survive, profound sequelae often affect physical and intellectual development [30]. Malarial sickness is one of the principal reasons for poor school attendance. Other high-risk groups are women during pregnancy, non-immune travelers, refugees, displaced persons and laborers entering endemic areas. Pregnancy-associated malaria is precipitated by the accumulation of parasites in the placental intervillous spaces and causes maternal anemia and low birth weight. It also is a major factor contributing to abortions and at times maternal deaths. Pregnant mothers who have malaria and are HIV-positive also are more likely to transmit HIV to their newborns. In sub-Saharan Africa, at least 25 million pregnant women are exposed to malaria every year.

Malaria therefore exacts an enormous toll in lives, in medical costs, and in days of labor lost [31]. For the individual, costs include the price of treatment and prevention, and lost income. In rural areas, the rainy season is often a time of intense agricultural activity, when poor families earn most of their annual income. Malaria can make these families even poorer, hitting young adults especially hard: a single bout of the disease costs an estimated equivalent of 10 working days. The estimated costs of malaria, in terms of strains on the health systems, are enormous: in endemic countries, as many as 3 out of 10 hospital beds are occupied by victims of the disease. In 2002, 40% of outpatients at Mozambique clinics and 60% of children admitted to hospitals suffered from malaria. The direct and indirect cost of malaria in sub-Saharan Africa is estimated to be 1–5% of GPD, a cost of about $12 billion a year [32,33].

3. Parasitology

The agents of human malaria are four species of \( Plasmodium \) protozoa: \( P. falciparum \), \( P. vivax \), \( P. ovale \) and \( P. malariae \) [4]. All are transmitted by \( Anopheles \) mosquitoes. Their only reservoir is humans, except for \( P. malariae \), which is common to man, the Africanapes and probably some South-American monkeys. Other species of \( Plasmodium \) are known (\( P. yoelii \), \( P. berghei \), \( P. chabaudi \), \( P. cynomolgi \)), that are adapted to their wild mammalian host and do not multiply in humans.

All strains of \( Plasmodium \) have a complex life cycle that begins when a female mosquito injects sporozoites into the blood of an individual when taking a blood meal. The sporozoites enter the bloodstream and within less than 30 min migrate to the liver and invade hepatocytes. Sporozoites mature in the liver over a period of 6–16 days. This period, known as the liver stage, or pre-erythrocytic stage, ends with the release into the blood of tens of thousands of merozoites, which invade RBCs where they multiply and mature over a period of a few days. There, they successively undergo a ring-form stage, a trophozoite stage and a schizont stage. The schizont eventually bursts, lysing the RBC and releasing new generation merozoites which immediately proceed to invade new RBCs to repeat the cycle. This is the blood stage or intra-erythrocytic stage of the disease, which is characterized by the classical acute febrile episodes that occur every 48–72 h, in coincidence with the synchronized lysis of infected RBCs and the release of newly matured merozoites.

Some of the merozoites eventually develop into sexual-stage gametocytes, which, after being taken up by an anophe-line mosquito, mature and sexually combine in the insect host to generate a zygote. The latter develops into an oocyst, from which new sporozoites are produced that migrate to the sali-vary glands of the mosquito, ready to reininitiate the cycle.

Pregnancy-associated malaria is caused by \( P. falciparum \), which expresses unique variant surface antigens (VSA) that allow the parasite to bind chondroitin sulfate proteoglycan receptors on the surface of placenta syncytiotrophoblasts [34,35].

The complete nucleotide sequences of the \( Plasmodium \) parasite vector has been determined. The \( Plasmodium \) genome is A–T rich, unlike most of the microbial organisms or animal cells used to express recombinant antigens, and it
also shows quite different codon usage. Optimal expression of recombinant *Plasmodium* antigens therefore requires the use of synthetic genes reconstructed with optimized codons best suited for the expression systems used for production.

4. Vaccines

4.1. General considerations

As mentioned above, several lines of evidence suggest that a prophylactic malaria vaccine for humans is feasible. Firstly, immunization of naive human volunteers with irradiated (and thus attenuated) sporozoites was shown to confer 90% sterile protection against experimental infection following laboratory-bred, sporozoite-infected mosquito bites [36,37]. Secondly, naturally acquired immunity progressively builds up during the first two decades of life in people living in malaria-endemic countries. This immunity primarily impacts the severity of clinical disease, and appears to be linked to continuous antigenic stimulation, waning rapidly when exposure ceases [10,38,39]. Thirdly, protection has been elicited by passive transfer of hyperimmune immunoglobulins from malaria-immune adults into malaria-naïve human volunteers [11].

Key obstacles to the development of a vaccine include the lack of immune correlates of protection, the lack of reliable and predictive animal models, and the developmental and antigenic variability of the parasite. An additional obstacle has been the paucity of expertise in GMP vaccine development in the non-industrial centers where much of the malaria vaccine research has been undertaken so far, and the lack of vaccine development platforms to develop clinically acceptable adjuvants able to induce cell-mediated immunity as well as epitope- or antigen-presenting systems. Much work has nevertheless been done to determine which protective antigens or epitopes should be used in the construction of recombinant, subunit or synthetic malaria vaccines.

The traditional approach to develop malaria vaccines has focused on the targeting of one of the different stages of parasite development, whether the pre-erythrocytic, the asexual (intra-erythrocytic) or the sexual stage. Pre-erythrocytic vaccine strategies aim to generate an antibody response able to neutralize sporozoites and prevent them from invading the hepatocyte, as well as to elicit a cell-mediated immune response able to interfere with the intra-hepatic multiplication cycle of the parasites, e.g. by killing the parasite-infected hepatocytes. This type of vaccine would be ideal for travelers because it would prevent the advent of any form of clinical disease. Asexual blood-stage (erythrocytic stage) vaccine strategies aim to elicit antibodies that will inactivate merozoites and/or target malarial antigens expressed on the RBC surface through antibody-dependent cellular cytotoxicity and/or complement lysis; and also are meant to elicit T-cell responses able to inhibit the development of the parasite in RBCs. By decreasing the exponential multiplication of merozoites, this type of vaccine would mostly serve as a disease-reduction vaccine in endemic countries [40]. As for vaccines that target the sexual stage of the parasite, they do not aim to prevent illness or infection in the vaccinated individual, but to prevent or decrease transmission of the parasite to new hosts. This ‘transmission-blocking’ vaccine can be seen as a true altruistic vaccine.

Besides these classical approaches, novel approaches being currently undertaken include the development of an irradiated sporozoite vaccine [41] and an anti-parasite toxin vaccine that targets the parasite toxins which contribute to the disease, such as the glycosylphosphatidylinositol (GPI) anchor. Recent promising results obtained with knock-out sporozoites in mice will likely lead to further developments over the coming years [21,42,43].

4.2. Pre-erythrocytic vaccines

These vaccines aim to protect against malaria infection [44] and ideally should provide sterilizing humoral immunity by eliciting antibodies that target the invading sporozoites and prevent them from invading the liver. Additionally, or as an alternative, the vaccine should induce a cell-mediated immune response able to inhibit the sporozoite maturation cycle once they have invaded hepatocytes [45]. CD4+ and CD8+ cytotoxic T cells (CTL), NK T cells, as well as γδ T cells have been implicated in such a control [46–51].

4.2.1. CSP vaccines

The most advanced and well-documented pre-erythrocytic (liver-stage) vaccine candidate is derived from the circumsporozoite protein (CSP) that is found at the surface of the sporozoite and of the infected hepatocyte. This protein contains two major B cell epitopes consisting of tandem repeats (Asn-Ala-Asn-Pro and Asn-Val-Asp-Pro). The candidate vaccine which was developed by Glaxo Smith Kline (GSK) in collaboration with the Walter Reed Army Institute of Research (WRAIR), RTS,S, comprises the C-terminus (amino acids 207–395) of the CSP from *P. falciparum* fused to the hepatitis B surface antigen and expressed in the form of virus-like particles (VLPs) in *Saccharomyces cerevisiae* [52,53]. Initial Phase I clinical trials of RTS,S formulated with GSK AS02 adjuvant (containing MPL, QS21 and an oil-in-water emulsion) showed protection against malaria challenge in six out of seven volunteers. A dose-range Phase I/II study showed levels of efficacy from 30% (single dose) to 55% (three doses), with an overall protective efficacy of 40%, but which, however, was not long-lived. Further trials in The Gambia demonstrated a 70% protection efficacy against infection for the first 9 weeks, with efficacy waning rapidly thereafter [54]. Volunteers who received a fourth dose of the vaccine the following year, prior to the onset of the malaria season, again exhibited a 47% protection over a 9-week follow-up period [53,55,56].

Clinical development of the RTS,S candidate vaccine has continued in partnership with PATH MVI. In a proof-of-
concept of three doses of RTS.S/AS02 at preventing a first malaria attack in 1–4 years old children was about 30% and, most significantly, overall incidence of severe disease was decreased 58% over a 6-month follow-up period [13]. Vaccine efficacy over a 18-month follow-up period was recently reported to be 35.3% against time to first or only episode of malaria and 48.6% against severe disease [14,57]. Other clinical studies of the RTS,S vaccine are underway, including one aimed at combining RTS,S with the blood-stage antigen MSP-1 (see below) [58,59]. Heterologous prime-boost immunization with RTS,S/AS02 and a live vaccinia virus (MVA)-CSP recombinant vaccine was well tolerated in human volunteers but yielded only small increments in T cell responses, no increase in antibody response and no improvement in percent protection [60].

The history of development of the RTS,S AS02 vaccine demonstrates the critical importance of appropriate adjuvants and formulations for the efficacy of vaccines based on purified antigens. Thus, the same malaria antigen formulated with aluminium hydroxide, with MPL, or as an oil-in-water emulsion failed to elicit a protective immune response, which was only obtained with the AS02 formulation, an oil-in-water emulsion plus MPL and QS21 [52]. The majority of other malaria recombinant antigens that have been clinically evaluated so far were formulated with either aluminium, QS21, or water-in-oil emulsions such as Montanide ISA 720, adjuvants which are less potent than the proprietary AS02 adjuvant and which may have been inadequate to induce protective responses in those studies.

Another CSP-based candidate vaccine was based on a 102-amino acid synthetic peptide, CS102, that represents the C-terminus of the CSP of *P. falciparum* formulated with Montanide ISA 720 [61]. The formulation, which was developed by Dictagen, Inc., in collaboration with the University of Lancaster, was shown to be safe in human volunteers and to elicit both an antibody and a cellular immune response including CD8+ CTLs and secretion of IFN-γ [62]. The vaccine now has undergone further Phase I and Phase IIa clinical trials, but has failed to show protection against experimental malaria challenge.

An alternative approach to synthetic vaccines was to incorporate several copies of a protective CSP epitope into a multiple antigenic peptide (MAP). As expected, the resulting vaccine was found to elicit an immune response only in the volunteers with cognate HLA haplotypes [63]. In an effort to bypass MHC restriction, the peptide was linked to a “universal” T-cell epitope. This construct was shown to elicit robust immune responses in humans with diverse genetic background [64]. The B- and T-cell epitopes from the MAP trials also have been incorporated into a recombinant VLP based on hepatitis B core particles. This CSP-HBc particle vaccine, known as ICC-1132, was developed by Apovia in the USA with the help of MVI. Although this candidate vaccine elicited both humoral and cellular immune responses, no evidence of protection was found in a Phase II study [65] and this approach was discontinued.

### 4.2.2. DNA vaccines and live recombinant vaccines

Other vaccines based on the CSP antigen include plasmid DNA vaccines and live recombinant vaccines that use the attenuated modified vaccinia Ankara (MVA) strain, fowlpox virus (FPV), Adenovirus, Sindbis virus, yellow fever virus or a cold-adapted attenuated influenza virus strain as a vector. Some of these vaccines were tested together in prime-boost combinations [66,67]. A series of trials using DNA and recombinant fowlpoxvirus or MVA vaccines encoding the *P. falciparum* CSP in various prime-boost combinations unfortunately did not show evidence of protective efficacy against a sporozoite challenge [68]. Both the MVA and fowlpoxvirus recombinant vaccines were nevertheless found to be safe and suitable for large scale studies of children in Africa [69,70].

In spite of promising humoral and cellular immune responses in mouse models, plasmid DNA vaccines injected through various routes did not translate into similar immunogenicity and protective efficacy in humans. The US Department of Defense (DOD), in collaboration with Vical, Inc., has developed candidate DNA vaccines for malaria (the Multi-Stage DNA Operation, MuStDO), including a liver-stage DNA vaccine that encodes the CSP of *P. falciparum*. This DNA vaccine was tested in a “proof-of-concept” Phase I study carried out by the US Navy Malaria Program. The vaccine elicited cell-mediated immune responses but only modest antibody responses and no protection against experimental challenge in human volunteers [71].

A multiple-antigen DNA vaccine, MuStDO-5, has been designed to encode five different liver-stage antigens: CSP, liver stage antigens 1 and 3 (LSA-1 and -3), exported protein 1 (EXP1), and the sporozoite surface protein 2 (SSP2, also known as thrombospondin-related adhesive protein, TRAP). Various studies conducted in endemic areas have linked LSA-1 and LSA-3 with protective immunity. MuStDO-5 was manufactured as a combination of five separate plasmids. The vaccine, administered with GM-CSF DNA as an adjuvant, was safe and well tolerated in mice and rabbits [72], but showed only weak immunogenicity in primates and no evidence of protection was obtained in Phase IIa challenge trials [73–75].

The Oxford University Malaria Vaccine Clinical Trials Group conducted studies of a DNA, a fowlpoxvirus (FPV), and a MVA-based vaccines expressing TRAP fused to a polyepitopic construct, demonstrating strong correlation between the induction of IFN-γ-secreting CD4+ and CD8+ T-cell responses and protection against malaria in a mouse model [76]. The DNA and MVA candidate vaccines were combined in a prime-boost immunization trial in human volunteers in The Gambia [77]. No protection was observed against occurrence of disease, but malaria-related mortality was reduced [75,77,78]. The substitution of plasmid DNA as the priming vector with a specific recombinant fowlpoxvirus vaccine was found to elicit sterile protection in two of five...
volunteers, and in one of them protection lasted for 20 months and was associated with persisting memory T cell responses [79]. The same recombinant FPV-TRAP/MVA-TRAP prime-boost immunization regimen was tested in Kenya. Strong T cell responses were elicited in children by half-dose FPV priming followed by MVA boosting [80], but the partial cross-reactivity between the two poxvirus vectors still might impair full immunogenicity of the MVA vaccine. A new efficacy trial is currently ongoing in Kenya.

Additional antigens that have been targeted for pre-erythrocytic vaccine development include the liver stage antigens 1 and 3 (LSA-1 and -3), the sporozoite and liver stage antigen (SALSA) and the sporozoite threonine and asparagine rich protein (STARP). LSA-3, a highly conserved pre-erythrocytic antigen, has been demonstrated to induce protective immunity against *P. falciparum* sporozoite challenge in chimpanzees and *Aotus* monkeys [81–84]. Various LSA-3 formulations, including a LSA-3 lipopeptide candidate are planned to presently enter Phase I trials.

### 4.3. Asexual blood-stage vaccines

These vaccines, also referred to as erythrocytic stage vaccines, are aimed to primarily protect against severe malaria disease, and not against infection, on the assumption that inhibition of parasite invasion cycles will lead to reduced parasite burden and decreased morbidity and mortality. Despite encouraging progress, the lack of immune correlates of protection and that of predictive animal models, together with the polymorphism and strain variability of many asexual stage antigens constitute major challenges to the development effort of asexual stage vaccines. In contrast with pre-erythrocytic vaccine candidates, asexual stage vaccine candidates also currently lack a human artificial challenge model and have to rely on natural challenge in field trials to provide proof-of-concept. Their development is therefore slower and necessitates major commitment, intensive collaborations as well as high-level coordination supported by adequate funding [22].

The first asexual blood-stage malaria vaccine to be developed was the SPf66 vaccine candidate that was developed in Colombia as a synthetic, multiepitope, multi-stage peptide vaccine mixed with alum as an adjuvant [85]. The vaccine was tested in several Phase III field trials involving thousands of volunteers, but its reported efficacy was too low to warrant further development [86–89], although one may suspect that the vaccine would have fared better with more potent adjuvants [90].

The most advanced asexual blood-stage vaccines at this time are based on the use of merozoite surface proteins 1 (MSP-1), 2 (MSP-2) and 3 (MSP-3), the apical membrane antigen 1 (AMA-1) and the glutamate-rich protein (GLURP). Antibodies to MSP-1 have been shown to block parasite invasion of RBCs *in vitro* [91]. AMA-1 is a natural target of protective responses *in vivo*. Both AMA-1 and MSP-1 have their 3D conformation stabilized by intramolecular disulfide bonds which are critical for optimal immunogenicity of the molecule. MSP-1 contains two cysteine-rich epidermal growth factor (EGF)-like domains that generate protective antibodies and are conserved across all species of *Plasmodium*.

#### 4.3.1. MSP-1, MSP-2 and RESA combination

Certainly the furthest along the vaccine development pathway of blood-stage malaria vaccine candidates is the “Combination B” vaccine, which resulted from a collaborative effort by the Papua New Guinea Institute for Medical Research along with the Australian Cooperative Research Center for Vaccine Technology in Queensland, The Walter and Eliza Hall Research Institute and the Swiss Tropical Institute. This vaccine combined the merozoite surface proteins MSP-1 and MSP-2 with *P. falciparum* ring-stage infected-erythrocyte surface antigen (RESA) in a Montanide adjuvant formulation. A Phase I/IIb trial in 120 5–9 years old children in Papua New Guinea showed a 62% reduction in parasite density in vaccinees [92]. However, the vaccine preparation contained only the 3D7 allelic form of MSP-2. Analysis of the genotype of breakthrough parasites showed a significant increase in parasites with the FC27 allele genotype, the opposite dimorphic form of MSP-2 [93]. A new version of the vaccine is being developed using both variants of MSP-2 in order to target both genotypes.

#### 4.3.2. MSP-1 and AMA-1

Much work has concentrated on the entire MSP-1 molecule, its 42 kDa C-terminal moiety, or a further-processed 19 kDa fragment [94]. These were expressed either as such or as part of fusion molecules using baculovirus, *Escherichia coli*, or yeast (*Saccharomyces* or *Pichia*). Recombinant MSP-1 42 kDa and 19 kDa fragments have been shown to protect both mice and *Aotus* monkeys against lethal parasite challenge [95–98], but a Phase I trial of the 19 kDa fragment carried out at Baylor University (USA) demonstrated that it was poorly immunogenic and had unacceptable side-effects. The MSP-1 42 kDa fragment formulated in AS02 adjuvant by GSK in collaboration with the Walter Reed Army Institute (WRAIR), USA, and MVI was found to be safe and very immunogenic in human volunteers in the USA, Kenya and Mali [99,100]. The vaccine currently is being tested in pediatric efficacy trials in Kenya. A fragment of the MSP-1 from *P. vivax*, termed Pv200L, which shows significant sequence homology with its *P. falciparum* equivalent, was found to provide partial protection against challenge with *P. vivax* in mice and *Aotus* monkeys [101].

Another vaccine studied in collaboration between WRAIR and GSK is based on the AMA-1 protein formulated in the AS02 adjuvant. Like MSP-1, the vaccine potential of AMA-1 is supported by the observation that antibodies to the protein inhibit invasion of RBCs *in vitro* and that recombinant AMA-1 protein can induce species-specific protection against malaria in rodent and non-human primate models.
[102,103]. The high polymorphism of the AMA-1 antigen is however a matter of concern [104–107]. An AMA-1 vaccine was studied in a Phase I study in the USA and now is in Phase I studies in Mali and Kenya [108,109]. A similar vaccine formulated in Montanide ISA 720 adjuvant was tested in a Phase I trial in Australia but only showed moderate immunogenicity [110].

A MSP-1/AMA-1 fusion antigen made of the C terminal region of AMA-1 and the 19 kDa fragment of MSP-1 [111] was produced in Shanghai using Pichia pastoris. The vaccine (PfCP-2.9), which was formulated with Montanide ISA 720 as an adjuvant, showed good immunogenicity in rabbits and non-human primates. WHO sponsored and coordinated the first Phase I clinical testing of the PfCP-2.9 vaccine in China in collaboration with the Second Military Medical University in Shanghai. The vaccine was found to be safe and immunogenic. Plans are underway for further clinical development through an agreement with MVI. The combination of the PfCP-2.9 vaccine with a fragment of the PfEBA-175 antigen expressed in P. pastoris yeast elicited in both rabbits and monkeys antibodies that inhibited parasite growth in vitro [112].

4.3.3. MSP-3, GLURP and SERA

The merozoite surface protein 3 (MSP-3), which was discovered by a reverse vaccinology approach and found to be a target of protective antibodies from immune adults, has been developed as a long synthetic peptide by the Pasteur Institute in Paris and the European Malaria Vaccine Initiative (EMVI). The vaccine construct contains B and T cell epitopes that were selected based on their targeting by cytophilic antibodies that interact with monocytes in the antibody-dependent cellular inhibition (ADCI) assay [113–116]. The African Malaria Network Trust (AMANET), in collaboration with EMVI, sponsored and coordinated a Phase I study of the vaccine in Burkina Faso, where the vaccine was shown to be safe and to induce long-lasting antibodies that display ADCI activity in vitro as well as in vivo in a new mouse model of P. falciparum malaria [117,118].

Another long synthetic peptide vaccine whose rationale is based on the induction of antibodies with ADCI activity is based on the glutamate-rich protein (GLURP), also a target of protective antibodies from immune adults [119–122]. The GLURP vaccine, which is developed by the Staten Serum Institute in Denmark in collaboration with EMVI, was formulated either in alum or Montanide ISA 720 and tested in a Phase I clinical trial [123].

Yet another candidate being developed in an Asian–African collaboration is based on the serine repeat antigen (SERA), also known as P126 antigen. This is the largest parasite protein, it accumulates in the parasitophorous vacuole of trophozoites and schizonts and is processed into three fragments (18 kDa, 47 kDa, 50 kDa) [124]. Cross-sectional studies conducted in the Solomon Islands, Brazil and Uganda [125] have shown a significant association between responses to SERA and lower parasitemia [126]. Various SERA-based constructs have been tested and shown to induce in vitro parasite killing activity in a dose dependent manner through both complement-mediated inhibition and ADCI [127,128]. Protective efficacy of a subunit SERA vaccine against challenge in Aotus and squirrel monkeys also has been demonstrated [129,130]. A candidate SERA-based vaccine currently is in Phase I studies in Japan.

4.3.4. Other antigens

Additional merozoite surface antigens under development as vaccine candidates include MSP-4, -5, -8 and -9. These molecules contain one or more of the hallmark EGF-like domains present in MSP-1 [131,132]. MSP-5 is of particular interest because it lacks the sequence variation between different isolates of P. falciparum from different geographical locations that is typically seen with most of the MSPs. The erythrocyte-binding antigen (EBA-175), which binds glycoerythrin A [133], and its paralog in P. vivax, the Duffy binding antigen (DBA), also are currently developed as recombinant vaccine candidates expressed either in E. coli or in P. pastoris or as a DNA vaccine in prime-boost regimens [134,135].

With regards to pregnancy-associated malaria, the erythrocyte protein 1 (PfEMP1) antigen, a highly variable P. falciparum protein, was identified as the ligand that allows infected erythrocytes to adhere to the chondroitin sulfate receptor. A candidate vaccine has been developed based on that antigen [136–138] but initial studies in Aotus monkeys failed to show protective efficacy [23].

4.4. Transmission-blocking vaccines

These vaccines are aimed to induce antibodies against the sexual stage antigens in order to prevent the development of infectious sporozoites in the salivary glands of the Anopheles mosquitoes [139]. They are intended to protect communities from infection, not individuals from disease and therefore face the difficult challenge of conducting Phase III trials to assess their impact on malaria transmission in the field. The leading candidate vaccines contain the P. falciparum ookinet surface antigens Pfs25 and Pfs28 or their P. vivax homologues Pvs25 and Pvs28 [135,140,141]. These vaccines are currently being developed at the National Institutes of Health (NIH) in the USA as recombinant yeast-secreted proteins (S. cerevisiae) [142,143]. Initial human Phase I trials that were conducted for Pvs25 demonstrated safety and a modest immunogenicity [144,145]. Other sexual stage-specific antigens that are being developed as transmission-blocking vaccines are Pfs48/45 and Pfs230 [146].

4.5. Other approaches

Various groups such as the Centers for Disease Control (CDC), Navy Medical Research Center (NMRC) and WRAIR are developing multi-antigens, multi-stage vaccine concepts [4]. Advantages of a combination vaccine include
the potential to address the problems of antigenic variation, of inducing immunity in genetically heterogeneous populations and of coping with possible immune escape of the parasite. However, the risk of interference between components of the vaccine and increased reactogenicity of the formulation must be taken into account.

The concept of attenuation and parasite challenge to elicit immunity also has been explored [42]. An attempt to develop an attenuated sporozoite vaccine has been undertaken by Sanaria, Inc., with the support from the Bill and Melinda Gates Foundation and the NIH. Challenges are numerous, including consistent production of large quantities of sporozoites, production quality control and process monitoring, GMP guidelines, stability, delivery and potential cost of the product [37,41] The possibility of using low doses of P. falciparum-infected RBCs also has been entertained [147].

Finally, the glycosylphosphatidylinositol (GPI) anchor, which tethers several of the Plasmodium antigens to the membrane, has been shown to be highly toxic in mouse models [148]. An anti-toxic vaccine is currently being developed as a carbohydrate anti-GPI vaccine. A proof-of-principle study testing synthetic GPI as a vaccine in rodent models of malaria showed that the candidate vaccine was immunogenic and protected the animals from significant malaria pathology and mortality [149]. Whether further development of malaria toxin neutralization as a vaccine strategy will continue is however far from certain.

5. Concluding remarks

The first stage in designing a malaria vaccine is the identification of the appropriate antigens and their formulation in a presentation that will elicit the desired protective immune responses. The lack of definitive knowledge on the nature of protective host responses to malaria deeply affects the assessment and rational prioritization of the current candidate Plasmodium antigens to be used in a malaria vaccine [150]. Since there are as yet no reliable correlates of protection, the selection of candidate antigens has been done by screening parasite extracts or gene libraries against sera or lymphocytes from immune individuals [151,152].

The determination of an antigen’s vaccine potential is highly dependent on its performance in biological assays that measure immunogenicity, yet the relevance of these immunogenicity studies often remains unclear. The paucity of standardized assays prevents useful comparisons between results from different laboratories often pursuing different formulations of the same antigen. Efforts to enable consistent intra- and inter-laboratory comparisons and thus to enhance the predictability of assay data are essential to provide confidence in decision-making relative to choice of candidate vaccines. An example of a standardized comparative immunoassay is the growth inhibition assay (GIA), which measures the capacity of antibodies to limit the growth of P. falciparum in RBCs in culture [153]. Another comparative assay is the ADCI assay, which initially was developed at the Pasteur Institute in Paris.

The various immune mechanisms of protection against malaria still are basically obscure [154]. Thus, vaccines against the pre-erythrocytic stage of the parasite have been developed with the goal of inducing antibodies that block invasion of hepatocytes by sporozoites, but field studies do not support a major role of these antibodies in protection. Antibody-opsonization of sporozoites, inhibition of intrahepatic parasite development by CD4+ and CD8+ T cell secretion of IFN-γ as well as killing of infected hepatocytes by CTL have been demonstrated to occur in response to the RTS,S vaccine [51] and in the irradiated sporozoite model [155]. Thus, current approaches aim to induce both these types of antibodies and T cell responses but the durability of the response remains a major challenge. Understanding the mechanisms by which CD4+ helper T cells are activated and the requirements for development of a specific and effective T cell memory and immunity is therefore essential in the quest of a malaria vaccine [156].

Regarding vaccines against the asexual (erythrocytic) stage of the parasite, multiple mechanisms have been identified which could underly protective immunity, such as antibodies that interact with the erythrocyte surface receptors and inhibit their invasion by merozoites, cytophilic antibodies that help destroy intra-erythrocytic parasites by monocytes, antibodies directed against merozoite surface antigens that mediate free merozoite agglutination and facilitate their phagocytosis, or antibodies that prevent the binding of infected erythrocytes to vascular endothelia, not to mention cytokines such as IFN-γ, TNF-α or IL-12, which can mediate parasite killing [157]. The major challenge met by vaccines which aim to elicit these types of responses is the high polymorphism and variability of the merozoite antigens [158].

As mentioned above, the absence of understanding regarding the nature of protective immunity adds to the complexity of vaccine development as the formulation of the parasite antigens remains purely empirical and a potentially protective antigen may fail in clinical trials because the formulation used may be inducing an inappropriate or inadequate immune response. The paucity of potent adjuvant systems with demonstrated clinical safety adds to the complexity of the field.

Another difficulty in the development of malaria vaccines stems from the absence of relevant animal models. Rodent models using P. yoelii, P. berghei or P. chabaudi, which lead to lethal infections, are basically different from the chronic infection with P. falciparum in humans [159]. The development of a SCID mouse model [160] might provide an interesting model. Non-human primates such as New World (Aotus or Saimiri) monkeys, rhesus macaques, Cercopithecus monkeys or chimpanzees are prime animal models for P. falciparum and P. vivax vaccine protection.
studies, but their cost, scarcity and strong ethical constraints limit their use. Also, the tolerance of high parasitemia and rapid acquisition of immunity following infection in the animals limit the interpretation of efficacy studies [161,162].

The choice of clinical case definitions and end-points in malaria vaccine trials is yet another difficult problem which may have marked influence on reported vaccine efficacy. Clinical case definition is straightforward for sterile protection, as used as an end-point in trials of pre-erythrocytic stage vaccines such as RTS,S [13] but can be most difficult for trials of vaccines which aim to decrease morbidity by reducing parasitemia [163].

All candidate malaria vaccines also meet with the problem of immunological memory. The protective immune status acquired through repeated natural exposure of infected individuals to the parasite over a period of years can readily be lost if continuous antigenic stimulation ceases. This suggests that low-level antigenic persistence is a prerequisite of immunity to malaria, and, therefore, that vaccines will need to simulate such a persistence to induce long-lasting protection [164]. So far, the hope was that boosting due to natural exposure would occur to provide this stimulus. Recent data suggest that alternating vectors in prime-boost vaccination regimens generate a large resting memory T cell population, which provides unprecedented durability of the immune response [79,165].

The major malaria vaccine funding agencies are the NIH in the USA, the Wellcome Trust in the U.K., the European Union, either directly through the European and Developing Countries Clinical Trials Partnership (EDCTP) or through the European Malaria Vaccine Initiative (EMVI) at PATH, the Bill and Melinda Gates Foundation and WHO. In addition, the African Malaria Network Trust (AMANET) and the Malaria Clinical Trial Alliance (MCTA) recently were established to build up the capacity to plan and coordinate malaria vaccine trials in Africa. The recent infusion of public and private funding for malaria vaccine development has greatly accelerated the pace at which candidate malaria vaccines are entering the clinic. Increased collaboration and cooperation among key research stakeholders in the malaria vaccine field also has played a role in this trend. A recent international consultative process that engaged vaccine researchers, experts and donors has resulted in a Roadmap, which outlines a strategy for the development and licensure of an effective pediatric malaria vaccine by 2015 and a more broadly effective vaccine by 2025. Top priorities were identified where intensified coordination and more focused action could be applied to overcome the significant challenges discussed above. The need for creative strategies, strong partnership between industrialized and developing countries and political vision and leadership on the part of wealthy nations is more than ever critical to successfully continue the development of malaria vaccines and implement their use in an effort to fight the terrible burden of malaria [166].

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