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Progress and Challenges towards the Development of Malaria Vaccines

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Abstract

The promise afforded by attenuated sporozoite vaccines in the 1970s led many researchers to believe that an efficacious malaria vaccine was an attainable medium-term goal. Over 30 years later, no licensed vaccine is currently available for public health intervention. This is despite global expenditure on research and development for malaria vaccines that is estimated to have increased from $US42 million in 1999 to $US84 million in 2004. Serious questions must therefore be asked: is this a good investment of research and public health funds, and are we really any nearer to producing a viable product for global use?

Proponents of a malaria vaccine promote this technology as a viable way to combat both the current economic and humanitarian burden of malaria and the decreasing efficacy of many front-line antimalaria drug therapies. The recent successful phase IIb trial of the RTS,S/AS02A vaccine showed that the production of a subunit vaccine with significant efficacy is technically possible. The combined efforts and financial commitment of researchers, pharmaceutical companies, and not-for-profit organizations, including the Malaria Vaccines Initiative, have resulted in a significant scaling up in the number of products suitable for testing in humans. In addition, new technologies, such as genetically attenuated vaccines and the exploitation of malaria genomes, offer exciting possibilities for vaccine development. There is now a real possibility of producing a malaria vaccine licensed for public health. However, this positive outlook must be tempered with the challenges facing vaccine development and distribution. The efficacy levels seen with RTS,S/AS02A are well below those of all vaccines currently in use for public health. Furthermore, poor preclinical and clinical predictors of efficacy, allele-specific immunity, and an imperfect understanding of natural and induced immunity to malaria may yet delay (or even prevent) the development of a vaccine suitable for global use.

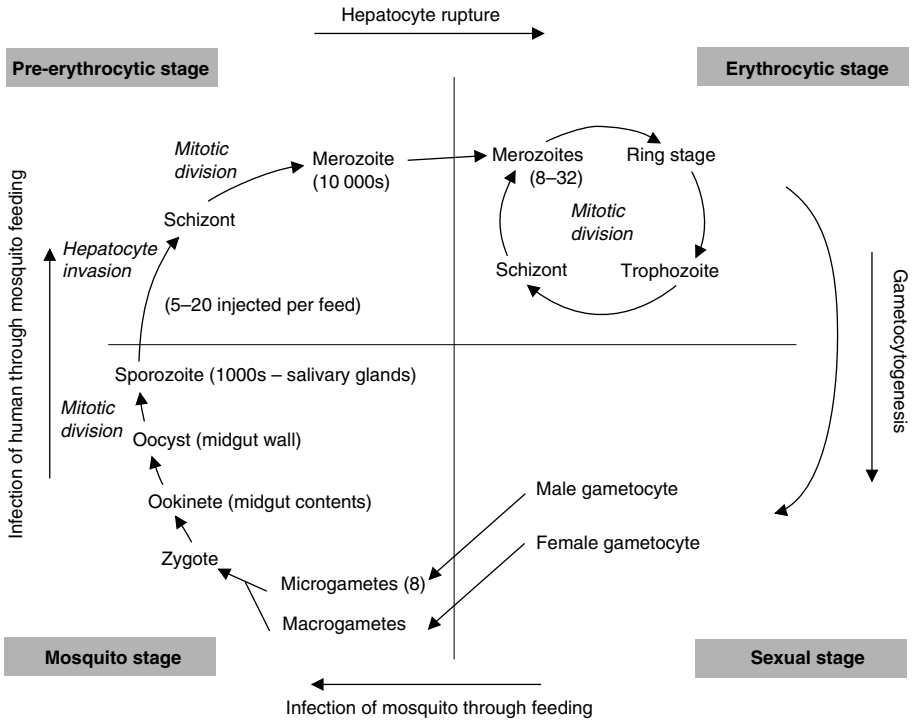

Fig. 1. Schematic of the *Plasmodium falciparum* life cycle: different stages to which a malaria vaccine can be targeted.

1. The Need for a Vaccine

With the exception of *Plasmodium knowlesi*,[1,2] there are four species of *Plasmodium* that infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. The majority of research and development into malaria vaccines is currently directed towards those products that would protect against *Plasmodium falciparum*, the most virulent of the human malarias.[3] This species is responsible for an estimated 300–500 million cases of clinical malaria and well in excess of 1 million deaths per year.[4] This in turn causes a major impact on the gross domestic product (GDP) of countries where malaria is endemic and the cost, in terms of lost productivity and medical care, exceeds $US1.7 billion each year. Sachs and Melaney[5] estimated that the gross national product per capita in malaria endemic countries has been reduced by >50% compared with non-malarious countries.

Globally, there is a marked increase in resistance to front-line antimalarial drugs, such as chloroquine[6,7] and sulfadoxine/pyrimethamine.[8] Even the newly licensed artemisinin derivatives run the risk of being rendered ineffective by the occurrence and spread of mutations within the *P. falciparum* genome.[9] Thus, there is a real need for novel intervention programs. However, were a successful vaccine to be licensed, it is uncertain whether a global market could effectively sustain such a product. Major concerns include the problem of a limited global vaccine production capacity and global production and distribution costs, together with an inability to deliver the vaccine to the target populations within existing infrastructures such as the Expanded Program on Immunization.[10,11] Nonetheless, the acceptance that these problems are insurmountable is not universal,[12,13] and many strategies have been proposed to overcome them. If the economics and logistics of vaccine manufacture, distribution, and delivery can be dealt with, then the advantages of an effective vaccine are clear.

2. What Stage Should a Malaria Vaccine Work Against?

A major factor when considering the component(s) to include in a malaria vaccine is the different outcomes that a vaccine can be devised to produce. Vaccines can be directed to prevent infection, reduce disease/parasite burden, or prevent the spread of infection from existing hosts depending on the stage of the *P. falciparum* life cycle to be targeted. As shown in figure 1, this life cycle can be divided into four main parts, three of which lie in the human host (pre-erythrocytic, erythrocytic, and sexual stages) while the fourth occurs within the mosquito vector.

Pre-erythrocytic-stage vaccines are designed to target either the sporozoites before they can invade hepatocytes, or the infected hepatocytes before they can produce infective merozoites.[14] It is at the pre-erythrocytic stage that the irradiated sporozoite vaccine works.[15-17] Such a vaccine would be expected to produce sterile immunity and would, theoretically, have little effect on the pro-

gression of disease once liver stage merozoites have successfully established an erythrocytic infection. Candidate antigens for such a pre-erythrocytic vaccine include those expressed in either sporozoites or within the hepatocyte, such as cirscumsporozoite protein (CSP) and liver stage antigen (LSA)-1, respectively.

By comparison, vaccines containing antigens expressed within the erythrocytic stage of the disease would be expected to reduce the overall parasite burden and associated morbidity.[18] Candidate parasite-derived antigens for erythrocytic-stage vaccines include merozoite surface protein (MSP)-1 (the major surface component of the merozoite), apical membrane antigen (AMA)-1 (released from the apical organelles of the merozoite prior to invasion and also expressed in the sporozoite stage), and *P. falciparum* erythrocyte membrane protein 1 (exported to the surface of infected erythrocytes and associated with parasite virulence). Individuals would most likely be rendered semi-immune, capable of maintaining infections without succumbing to clinical episodes of the disease. An alternative strategy is to induce immune responses to detoxify by-products of infection, such as glycosylphosphatidylinositol, which may play a role in the severity of clinical morbidity.[19]

Finally, transmission-blocking vaccines have been promoted as a way to interrupt the sexual stage of the life cycle. The vaccine would not directly protect an inoculated individual, but rather act to reduce the rate at which new infections are established via the mosquito vector, thereby reducing overall disease rates in endemic regions. Such actions would undoubtedly complement the effects of insecticide-treated bed nets and residual spraying. Transmission-blocking vaccines would induce antibodies against antigens expressed during the sexual stages of the malaria parasite, either on the gametocytes resident within the human blood stream or, in the case of the Pfs25 protein, in the stages found within the mosquito mid gut.

One possible way forward is to include multiple antigens within a vaccine in order to replicate the activity of all three vaccine types. However, the inclusion of multiple antigens within a single vaccine may be hampered by the higher development and production costs to produce a homogeneous product and ensure that no interference occurs between the different antigens.

3. Irradiated Sporozoites and Subunit Vaccines

In the early 1970s, sterile immunity was achieved in naive volunteers using the bites of irradiated mosquitoes infected with *P. falciparum*. Using this approach, ≤90% sterile protection was achieved against experimental infection.[15-17,20] Unfortunately, the logistics required to produce infected irradiated mosquitoes suitable for a global vaccination program has so far precluded this approach as a viable public health intervention strategy. Even if it were possible, the requirement for thousands of infected bites makes it unfeasible to advocate such a vector-based approach in the field.

Given the problems associated with an irradiated sporozoite vaccine, subunit vaccines have been explored as possible alternatives. Human and animal subjects presenting with sterile immunity due to irradiated sporozoite inoculation appear to show elevated T cell-mediated responses to a limited number of *P. falciparum* antigens.[17,20-23] Thus, there is a real possibility that subunit vaccines using a defined panel of polypeptides could induce protective sterile immunity.

Such immunity would be different from that presented by people living in areas where malaria is hyperendemic, such as The Gambia. People in these locations develop the capacity to maintain low-level infections without associated clinical disease over the course of repeated infections, with the greatest burden of malaria being carried by children <5 years of age.[24] The requirement for repeated infection is such that in areas of low endemicity no effective immune response is generated, resulting in an equal burden of disease in both adults and children. The humoral component of this immunity has been demonstrated by the passive transfer of immunoglobulin G (IgG) from healthy semi-immune African volunteers to non-immune children infected with *P. falciparum*, resulting in an accelerated clearance of clinical morbidity.[25,26] It has been shown that people living in locations endemic for malaria possess cellular reactivity to specific malaria antigens[27] and some of these are associated with protection.[28] However, with a lack of antigen processing in erythrocytes the majority of immunity to blood-stage malaria parasites will most likely be conferred by humoral immune responses. Any malaria vaccine formulated to mimic natural immunity against blood-stage parasites would be expected to elicit high titers of antibodies against specific antigenic targets that are capable of controlling an infection. However, the specific mechanisms of protection and how to measure them are poorly understood, limiting the quality of information that can be used to guide vaccine design.[24]

The major problem with subunit vaccines containing recombinantly produced polypeptides is the limited number that can currently be accommodated within a single vaccine. The first licensed subunit vaccine, developed for hepatitis B, contained a single hepatitis B surface antigen (Recombivax HB®,[1] Merck & Co Inc.).[29] Although vaccination with DNA-based constructs offer the potential to overcome these limitations by the incorporation of epitopes from multiple antigens, success with these systems has

1 The use of trade names is for identification purposes only, and does not imply endorsement.

been very limited. Recently, Dunachie et al.[30] showed that a prime boost regimen using TRAP (thrombospondin-related anonymous protein) but not CSP produced partial protection in healthy, malaria-naive adults. However, recent vaccine trials have failed to show protective efficacy in either Gambian adults[31] or Kenyan children.[32] This is despite strong immunogenicity data with relation to induction of effector T-cell responses in volunteers.[31]

Subunit vaccines that are produced by fragmenting whole parasites may contain many more components but require the capacity for large-scale culture of the infectious organisms. For example, the Pneumovax® II (Sanofi Pasteur MSD) pneumococcal vaccine contains capsule polysaccharides from 23 common types of *Streptococcus pneumoniae*. The major problem for subunit vaccines remains which (if any) out of the predicted 5000 plus components of the *P. falciparum* proteome in isolation are likely to induce an effective immune response capable of combating infection. It is sobering to note that this is a parasite that has been shaped through evolution to evade an immune system that is exposed to its full antigenic repertoire during the course of a natural infection.[33] The quest to answer this question has seen the development of numerous preclinical assays to determine the suitability of a candidate antigen as a vaccine component.

4. Preclinical Analysis of Candidate Antigens for a Vaccine

Table I provides a summary of candidate antigens currently under investigation for use in subunit vaccines. It can be seen that there are numerous lines of evidence that can be used to support development of a particular candidate to a good manufacturing practice (GMP) product suitable for use in human trials. These include *in vitro* invasion inhibition (with both hepatocyte and erythrocyte models), cytoadherance inhibition, antibody-dependant cellular inhibition (ADCI), immuno-epidemiology studies, opsonization studies, and vaccination of animal models. For example, the addition of anti-AMA-1 antibodies to *in vitro* cultured malaria parasites causes a significant decrease in the asexual growth rate, interfering with the erythrocyte invasion process.[34] In addition, immuno-epidemiology studies with recombinant AMA-1 have shown a significant association of naturally occurring anti-AMA-1 antibodies with reduced occurrence of clinical disease in the subsequent transmission season in Kenyan children.[35] Finally, immunizations in animal model systems with AMA-1 (alone or as a hybrid) preclincial vaccines have elicited immune responses capable of either preventing or limiting experimental infections,[36,37] supporting their development to GMP products.

While positive results with preclincial assays suggest that creating an efficacious malaria vaccine is possible, there are problems inherent in the interpretation of such data that have prevented any one assay being universally accepted as a 'gold-standard' appropriate for all candidates. Furthermore, there is no standardized criterion that would allow a like-for-like comparison of candidates analyzed by different methodologies (table I). It is difficult therefore to prioritize candidacy of different antigens, especially when assays may give conflicting results; purified human IgG that is capable of reducing clinical morbidity in passive transfer experiments has no effect on invasion rates of parasites *in vitro* but is associated with ADCI.[120,141] Even within a single assay, such as *in vitro* invasion inhibition, there is often no standardized cut-off constituting a positive result. Additionally, different immuno-epidemiologic studies will often yield conflicting results for a given antigen, perhaps as a result of differences in the ethnicity of the cohorts, malaria transmission, or misclassification of an individual's immune responsiveness.[24]

For animal immunization models there are also drawbacks, making it impossible to guarantee how a vaccine candidate will function within an endemic location. In the mouse model system, the rodent malaria parasites *P. yoelii* and *P. berghei* are often used to analyze the homologs of *P. falciparum* vaccine candidates. However, there is a predicted evolutionary distance of >60 million years between these species of malaria.[142] The effect of this divergence is that direct homologs are not always identifiable (as seen with many of the *P. falciparum* erythrocyte binding antigen genes).[143] Even when the homolog is readily identifiable there is no guarantee the identified genes would have an identical function in the two species, as shown by gene knockout and transcriptional studies (S. Polley, unpublished). It is also of interest to note that mice are not the natural host of these parasites and may not be the best model organisms to use if a natural infection is to be studied. Mice have a strong innate immune response against *P. berghei* not seen in the natural host *Grammomys surdaster* (thicket rat).[144] A severe combined immunodeficiency disease (SCID) mouse model with humanized erythrocytes offers the ability to use *P. falciparum*;[145] however, even this system is hampered as a result of the differences that exist between the human and murine immune system.

The use of monkey and primate models offers a closer approximation to the human immune system for preclinical safety and efficacy studies. The common model organisms are *Aotus* and rhesus monkeys together with splenectomized chimpanzees. The *Aotus* monkey model system offers the flexibility to use erythrocytic challenge with strains of *P. falciparum* that have been adapted to this host;[146] in addition, certain *Aotus* species have the ability to be infected with sporozoites from infectious mosquito

Table I. Summary of potential malarial vaccine candidates currently under investigation

Stage-specific candidates (expected outcome)	Supporting experimental evidence for candidacy			
	protection/transmission-blocking in experimental infection[a]	immuno-epidemiology	invasion/growth inhibition	antibody-dependent cellular inhibition
Liver stage (anti-infection)				
CSP-1	+[21,38,39]	+[40,41]	+[42]	
TRAP	+[40,43,44]	+[40,45,46]		
STARP			+[46]	
LSA-1		+[28,40,47-49]		+[50]
LSA-3	+[51,52]			
EXP-1		+[53]		
Erythrocytic stage (anti-disease)				
MSP-1	+[54,55]	+[41,56,57]	+[54,57-60]	
AMA-1	+[36,61-65]	+[35]	+[58,60,66-70]	
EBA-175	+[71-73]	+[74]	+[41,71,75,76]	
RAP-1	+[77]	+[78-80]	+[77,81-84]	
RAP-2		+[80]	+[81]	
SERA-5		+[85,86]	+[87,88]	+[89]
MSP-2		+[90,91]		
MSP-3	+[92]	+[53,93,94]		+[95,96]
MSP-4	+[97-100]	+[101]		
MSP-5	+[97-100]			
PfEMP-1	+[102-106]	+[107-110]	+[111,112]	
GLURP	+[92]	+[53,94,113-118]	+[119]	+[120,121]
RESA	+[122,123]	+[113,124,125]	+[126,127]	
Sexual stage (anti-transmission)				
Pfs48/45	+[128]	+[128-130]		
Pfs230	+[131]	+[130,132,133]		
Pfg27	+[134]			
Pfs16			+[135]	
Pfs25/28	+[134,136-140]			

a In humans or animals. In sexual stages, transmission-blocking activity prevents infection of the mosquito vector.

AMA = apical membrane antigen; **CSP** = circsumsporozoite protein; **EBA** = erythocyte binding antigen; **EXP** = exported protein; **GLURP** = glutamate-rich protein; **LSA** = liver stage antigen; **MSP** = merozoite surface protein; **PfEMP** = *Plasmodium falciparum* erythrocyte membrane protein antigen; **Pfg** = *Plasmodium falciparum* genetocyte antigen; **Pfs** = *Plasmodium falciparum* sexual stage antigen; **RAP** = rhoptry associated protein; **RESA** = ring-infected erythrocyte surface antigen; **SALSA** = sporozoite- and liver stage antigen; **SERA** = serine repeat antigen; **STARP** = serine threonine asparagine rich protein; **TRAP** = thrombospondin-related anonymous protein; **+** indicates evidence has been reported.

bites.[147] However, the progression of disease in *Aotus* species deviates from that seen in humans in a number of ways. These include a tendency to life-threatening anemia and a rapid acquisition of effective blood-stage immunity.[148] In addition to this, there are substantial differences between human and *Aotus* major histocompatibility complex gene sequences. Although genetically closer to humans than *Aotus* monkeys, the rhesus monkey system is refractory to *P. falciparum*, necessitating the use of homologous malaria parasites (*P. knowlesi* and *P. cynomolgi*) with the associated problems due to evolutionary divergence.[148] Even the chimpanzee (our nearest genetic relative) shows substantial differences in its susceptibility to *P. falciparum*, most likely due to alterations on the erythrocyte surface.[149] These potential draw backs, along with substantial financial, ethical, and time consideration associated with monkey trials, have prompted some to question the utility of this methodology.

Table II. Steps in the vaccine development pipeline[152]

Program/trial stage	Objectives	Duration	Population	Sample number
Basic research	Antigen identification	NA	NA	NA
Applied research	Concept	NA	NA	NA
Preclinical development	Evaluation in animals	NA	NA	NA
Process development	Creation and validation of manufacturing process	NA	NA	NA
Phase Ia	Safety and immune response	≤12mo	Non-endemic	<30
Phase Ib	Safety and immune response	≤12mo	Endemic	<100
Phase IIa	Safety, immune response, and preliminary efficacy	≤12mo	Non-endemic	<30
Phase IIb	Safety, immune response, and preliminary efficacy determination of dosage	≥2y	Endemic	100–1000
Phase III	Safety and efficacy	3–5y	Endemic	>10 000
Licensure	Regulatory approval for distribution	NA	NA	NA
Introduction	Lives saved	NA	NA	NA
Phase IV	Follow-up safety and effectiveness	4–6y	Endemic	>100 000

NA = not applicable.

Given the heterogeneous outcomes achieved with the current preclinical models, it is hardly surprising that 2 of the 11 goals of the Malaria Vaccine Technology Roadmap[150] are to improve the understanding of correlates of protection and to establish a systematic approach for prioritizing subunit vaccine candidates using accepted preclincial criteria.

5. The Pipeline for Development

Table II shows the stages a promising vaccine candidate must go through to arrive at a finished product licensed for use in humans. The current total development cost of a single pharmaceutical product is around $US200–800 million over a 7- to 14-year period, and it is unlikely that the costs for a malaria vaccine will be significantly lower. This requirement is a serious limitation to the number of vaccine candidates that can be taken forward, even in the current environment of increased funding. The Bill and Melinda Gates Foundation, a significant financial contributor to malaria vaccine development, admit that the process is complicated, "and more expensive than we anticipated."[151] To proceed to any clinical trials (phase I onwards), the product must first be manufactured to a strict set of guidelines, set down as GMP. The limited global capacity for GMP production is another cap on the number of products that can advance to clinical trials. Addressing this is also a major goal of the Malaria Vaccine Technology Roadmap. Companies in locations such as India and China offer a viable opportunity to expand GMP production and fast track a number of promising candidates through to clinical trials.

A major problem when considering erythrocytic vaccines is the inability to perform experimental human challenge with blood-stage parasites when assessing the protective efficacy of blood-stage vaccines in phase IIa trials. Although the 3D7 strain is licensed for human experimental infection, there is no standardized measure of correlates of protection when a delay in clinical presentation of disease is the endpoint. In contrast, presence or absence of blood-stage infection as a marker of sterile immunity is a well established endpoint for pre-erythrocytic vaccines.[15] However, even with pre-erythrocytic vaccines, experimental infection can be a poor approximation for natural infection in an endemic location, which is illustrated by the conflicting results obtained with the multiple epitope (ME)-TRAP vaccine when analyzed with experimental (phase Ia) and natural (phase Ib) infections.[32,153] Could this be due in part to the nature of the 3D7 strain licensed for experimental infections? This parasite clone has undergone numerous passages since it was first adapted for *in vitro* culture. It has been shown that the parasite's genome has undergone rearrangement and loss of material with unknown consequences for the infectivity or virulence of the parasite.[154,155]

A third limitation on the numbers of vaccine candidates that can be pushed through this pipeline is the number of people needed at each stage of the development process (see table II). This is affected by whether the required endpoint of the trial is protection against infection or protection against clinical manifestation of disease (cohort sizes are significantly larger for the latter).[12] The logistics involved in recruiting volunteers, administering the vaccination programs, and collecting endpoint measurement means that there are only a limited number of locations where the phase

IIb and III studies can be implemented. This is because the required infrastructure is often lacking in locations endemic for malaria. A recent venture to establish an HIV vaccine trial in Haiti highlights some of the logistical problems associated with vaccine research in developing countries.[156] These include a weak health infrastructure, a shattered economy, high unemployment, residual political instability, and a high rate of illiteracy, as well as daily logistical hurdles like bad roads, erratic telephone networks, and energy blackouts as some of the problems facing would be trial administrators.

Another goal of the Malaria Vaccine Technology Roadmap is the scaling up of such vaccine trial facilities, although it is also important to remember the ethical as well as logistical considerations of such trials when planning them. Ethical considerations include the vulnerability of child participants, likely benefits versus risks to the research subjects, ability to ensure informed consent, standard of care for research subjects should the intervention fail, and access for the subjects and community to the product given a successful intervention. One ethical consideration particularly relevant to malaria vaccines is the issue of safety versus efficacy of the product. Could more lives be saved with a product that was more efficacious but caused more severe adverse effects? In addition to these concerns, communities involved in such interventions can easily develop research fatigue if they come to view themselves as mere guinea pigs for clinical research and experience a lack of positive outcomes with the trials.[157]

A further complication with trials involving humans is the choice of adjuvant incorporated with the vaccine antigen(s). These immunomodulators include a variety of compounds, which are designed to enhance the immunogenicity of antigens, thereby magnifying, accelerating, and prolonging the immune response. Adjuvants licensed for use in humans include mineral salts such as the commonly used alum, microbial derivatives such as monophosphoryl lipid A, oil-in-water emulsions such as Montanide ISA-51, and the proprietary adjuvant AS02A. The choice of adjuvant can have a major effect on the performance of a vaccine, as shown by a recent trial of malaria vaccine in treatment-naive volunteers.[158] In this study the same recombinant protein was conjugated with three different adjuvants as follows:

- vaccine 1 – alum and monophosphoryl lipid A
- vaccine 2 – an oil-in-water emulsion
- vaccine 3 – an oil-in-water emulsion plus monophosphoryl lipid A and QS21.

Vaccines 2 and 3 elicited significantly higher titers of antibodies against the recombinant protein than vaccine 1, while only those volunteers given vaccine 3 demonstrated a high (>80%) level of protection against subsequent experimental infection. However, with many human licensed adjuvants being poor immunomodulators in animal models,[159] it is only at the stage of human testing that antigen/adjuvant combinations can be assessed.

6. The Success of the RTS,S/AS02A Vaccine

Table III provides a summary of malaria vaccines that are currently under development for clinical trials. This list includes subunit vaccines based on recombinant polypeptide products conjugated with a suitable adjuvant and DNA-based vaccine constructs (such as ME-TRAP).

As can be seen from table III, only a very limited number of products have made it through to any kind of efficacy testing (phase IIb or higher) in an endemic location. SPf66 (omitted from table III) was the first malaria vaccine to be taken to phase III trials. However, despite promising results from four South American trials with SPf66, these trials produced disappointing results when conducted in Africa. For instance, an efficacy of only 2% was seen in a Tanzanian trial of children in their first year of age, resulting in SPf66 being abandoned by many research agencies as a viable vaccine[195] and causing a considerable degree of controversy.

The disappointment of SPf66 has been superseded by a successful phase IIb trial of the RTS,S/AS02A vaccine. This vaccine has been developed by GlaxoSmithKline (GSK) Biologicals in combination with the Walter Reed Army Institute of Research (WRAIR). It is comprised of the CSP antigen repeat region (R) and T cell epitopes (T) coupled to the core of the licensed surface antigen (S) subunit hepatitis B vaccine conjugated with proprietary adjuvant AS02A. An RTS,S/AS02A trial was carried out in Mozambique on 1142 children, aged 1–4 years old, who received a 3-dose regimen of the vaccine with no booster in the follow-up period. The immunized children showed a reduction in clinical malarial episodes of 35% and severe malaria episodes by 49% in the 18-month period of follow-up, compared with control subjects.[161] An even greater (58%) reduction in severe malaria was seen in the initial 6-month follow-up period.[196] This trial has raised hopes that an efficacious malaria vaccine that will induce long lasting immunity is technically achievable.

7. Allele-Specific Immunity

A major barrier to malaria vaccine development is the polymorphic nature of many of the vaccine candidates. Even the CSP antigen is polymorphic, with an over-representation of polymorphic residues within sites shown to stimulate a T-cell response.[197] If the regions of antigens to be used in a vaccine are polymorphic in nature, then a suitable diversity of allelic forms must be included to protect against the total diversity encountered through natural infection (strain-transcending immunity). Failure

Table III. Summary of malarial vaccine candidates in production[160]

Vaccine	Company or research group	Delivery systems/technology	Development phase	References
Pre-erythrocytic stage				
RTS,S	GlaxoSmithKline, Walter Reed Army Institute of Research (Silver Spring, MD), Malaria Vaccines Initiative (Bethesda, MD)	Recombinant: CSP-HBs	IIb	158,161-166
CSP C-term	Dictagen Inc., Lausanne University (Switzerland)	Peptide	Ib	167,168
ICC-1132	Apovia Inc. (San Diego, CA), Malaria Vaccines Initiative (Bethesda, MD)	VLP: CSP-HBc	II	169-171
MuStD0-5 (various)	US Navy, Vical Inc. (San Diego, CA)	DNA: CSP ME-HBc	I	172
LSA-1	Oxford University, Oxxon Therapeutics, Inc. (UK), Malaria Vaccines Initiative (Bethesda, MD)	Prime-boost: FPV or MVA	Ib	173
ME-TRAP	Oxford University, Oxxon Therapeutics, Inc. (UK)	Prime-boost: MVA	Ib	31,174-176
CSP	Crucell (Leiden, The Netherlands), GlaxoSmithKline, Walter Reed Army Institute of Research (Silver Spring, MD), National Institute of Allergy and Infectious Diseases (Bethesda, MD)	Prime-boost: adenovirus	Ia	177
CSP	Oxford University (UK), New York University (New York)	Prime-boost: MVA, cold adapt	Preclinical	178
LSA-3	Pasteur Institute (France), Walter Reed Army Institute of Research (Silver Spring, MD), GlaxoSmithKline	LSP/LPP/recombinant	Preclinical/IIa	51,179
Erythrocytic stage				
MSP-1 19/EBA-175/MSP-1 19+EBA-175 F1	International Centre for Genetic Engineering and Biotechnology (Trieste, Italy), European Malaria Vaccine Initiative (Copenhagen, Denmark)	Recombinant	Ia	180
MSP-1-42	GlaxoSmithKline, Walter Reed Army Institute of Research (Silver Spring, MD), Malaria Vaccines Initiative (Bethesda, MD)	Recombinant	Ib/II	59,181
AMA-1	National Institute of Allergy and Infectious Diseases (Bethesda, MD)	Recombinant	Ib	182
PfCP 2.9	Second Military Medical University (Shanghai, China), Wanxing Bio-Pharmaceuticals Co., Ltd (Shanghai, China), World Health Organization	Recombinant subunit (yeast): AMA-1-MSP-1	I	60
MSP-1 derivatives	National Institute of Allergy and Infectious Diseases (Bethesda, MD), Hawaii Biotech, AECOM, University of Maryland	BCG recombinant subunits	Preclinical/I	183-186
MSP-3	Pasteur Institute, AMANET, European Malaria Vaccine Initiative (Copenhagen, Denmark)	LSP	I	187

Continued next page

Table III. Contd

Vaccine	Company or research group	Delivery systems/technology	Development phase	References
GLURP	European Malaria Vaccine Initiative(Copenhagen, Denmark), Statens Serum Institut (Copenhagen, Denmark)	LSP	I	119
MSP-3-GLURP	European Malaria Vaccine Initiative (Copenhagen, Denmark), Statens Serum Institut (Copenhagen, Denmark)	LSP	I	94,188,189
MSP-4, -5	Monash University (VIC, Australia)		Preclinical	100
Combination B	Queensland Institute of Medical Research (Australia), La Trobe University/The Walter and Eliza Hall Institute for Medical Research (WEHI) [Melbourne, Australia]	Recombinant	II	190,191
SE36 (SERA-5)	Osaka University (Osaka, Japan)	Recombinant	I	85,192
Transmission-blocking				
Pfs25 (Pvs25; various)	National Institutes of Health (USA)	Recombinant	Preclinical/I	136,137,193,194

AMA = apical membrane antigen; **BCG** = Bacille Calmette-Guérin; **CSP** = circumsporozoite protein; **DNA** = DNA vaccine; **EBA** = erythocyte binding antigen; **FPV** = fowl pox vaccine; **GLURP** = glutamate-rich protein; **HBc** = hepatitis B core subunits; **HBs** = hepatitis B surface antigen; **LPP** = lipopeptides; **LSA** = liver-stage antigen; **LSP** = long synthetic peptides; **ME-TRAP** = multiple epitope thrombospondin-related adhesion protein; **MSP** = merozoite surface protein; **MVA** = modified vaccinia Ankara; **PCCP** = *Plasmodium falciparum* chimeric protein; **RTS,S** = repeat region, T cell epitopes, and hepatitis surface antigen; **SERA** = serine repeat antigen; **VLP** = very long peptides.

to do this will most probably result in the selection of divergent variants, rapidly reducing any efficacy that a vaccine may have initially had. The problem posed by polymorphic candidate antigens is highlighted by the outcome of a phase IIb trial with the Combination B vaccine in Papua New Guinea.[190] While no significant protection was seen, there was an apparent skew in the *Msp2* gene frequencies within immunized volunteers, such that the frequency of heterologous allele type increased significantly in the subsequent transmission season. The Combination B vaccine is based around three *P. falciparum* merozoite- and ring-stage derived recombinant protein antigens (MSP-1/MSP-2/RESA) formulated with Montanide ISA 720 as adjuvant. MSP-2 is an antigen with a highly polymorphic structure, with *Msp2* alleles grouping into two major allelic classes (IC-1 and Fc27). The failure of Combination B would appear to the result of induction of allele-specific rather than strain-transcending immunity. Such a phenomenon has been observed in experimental challenges in animal model systems and *in vitro* growth inhibition assays, whereas allele-specific antibodies are commonly displayed by people in areas where malaria is endemic.[35,56,93,198]

Including multiple allelic forms of a given antigen will undoubtedly increase the complexity, development time, and overall cost of a vaccine. Tetteh et al.[199] demonstrated the effectiveness of using an epitope-mapping strategy in the rational design of a multi-allelic construct comprising highly polymorphic structures, such as those found in the N-terminal MSP-1 block 2 region,[56,200] to induce immunity against a broad spectrum of genotypes. Franks et al.[201] showed that despite the presence of significant polymorphic differences between different members of the same MSP-2 allelic class, conserved epitopes within the IC-1 type (and to a lesser degree the Fc27 type) can elicit antibodies that bind to all antigens of a given allelic type. A rational approach to vaccine design may therefore be capable of limiting the number of allelic forms needed to induce strain-transcending immunity. The ability to genetically engineer different allelic sequences into long chimeric proteins offers the ability to overcome gene diversity. This approach has allowed the development of a recombinant polypeptide vaccine against the diverse group A streptococcus, a causative agent of life-threatening necrotizing fasciitis, toxic shock, and rheumatic fever.[202] Encouragingly, initial trials showed no selective change in allele frequencies induced by the RTS,S/AS02A vaccine in Mozambique, suggesting that allele-specific immunity was not a problem when using this vaccine,[203] despite the polymorphic nature of CSP-1.

An alternative approach to using divergent allele sequences is to concentrate on those antigens/antigenic regions that are conserved amongst isolates. Anti-MSP-3 antibodies were first identified as correlates of immunity through passive transfer studies.

The N-terminal region is highly polymorphic (with sequences clustering into two main allelic types) and allele-specific immune responses to this region have been shown to be associated with protection.[93] It could be argued, therefore, that an MSP-3-based vaccine should contain both sequence types to induce strain-transcending immunity. The Pasteur Institute (Paris, France) has instead looked to construct a vaccine containing only the relatively conserved C-terminal region of MSP-3.[204] In human volunteers, this vaccine has been shown to induce antibodies capable of clearing infection in the humanized SCID mouse model,[204] raising hopes for its efficacy and proving the utility of such an approach. It is important to consider why certain antigen regions, such as the C-terminus of MSP-3, show little or no diversity in the wild. It is possible that these sequences may not be under sufficiently strong immune-selection pressure to maintain polymorphisms in natural populations. Alternatively, they may be under such strong purifying selection that mutations can not accumulate without causing a serious deleterious effect on the parasites fitness. It is important to note that under novel positive-selection pressures, even sequences subject to purifying selection can accumulate polymorphisms that would normally be deleterious. This has been seen in HIV infections from patients undergoing antiretroviral therapy, and poses a serious limit to the lifespan of front-line treatments.[205]

8. Genetically Attenuated Vaccines

A number of groups have recently developed attenuated whole-organism vaccine candidates in an attempt to replicate the immunity induced by irradiated sporozoites. Van Dijk et al.[206] described the genetic attenuation of *P. berghei* sporozoites by disruption of the *p36* gene, which encodes a member of the P48/45 family of sporozoite surface proteins. When infected into mice, the attenuated parasites invaded hepatocytes but failed to develop beyond this stage. These arrested infections induced immune responses capable of protecting against subsequent experimental challenge with non-attenuated parasites. More recently, Mueller et al.[207] showed that disruption of the *UIS3* gene gave rise to genetically attenuated parasites that were incapable of developing into liver stage merozoites and, therefore, did not lead to disease. Immunization of mice with the *uis3*(–) parasites led to a sustained, stage-specific protection. Although questions of safety and potency need to be addressed,[208] it is worth noting that neither of the early phase studies for RTS,S or the DNA-based vaccines demonstrated the level of protection observed following immunization with irradiated sporozoites.[207,208] As with irradiated sporozoites, the attenuated vaccine approach can only be viable for global vaccination if sufficient material for large-scale immunization can be produced in culture and a suitable delivery system (preferably not vector-based) can be developed for human use.[209]

9. Current and Future Developments in Malaria Vaccines

Many of the vaccine candidates in table I were initially identified based on the reactivity of human sera against them (due to either natural infection or experimental sporozoite immunization) or an abundance of the antigen in fractionated parasite extracts. The analyses of these candidates through classical techniques (such as cloning from bacterial expression libraries or purification and protein sequencing) were time consuming, resulting in a slow (but steady) progress towards defining candidates antigens. Novel approaches in identifying new vaccine candidates and speeding up their molecular characterization will greatly increase the number of candidates brought to preclincial analysis. If this can be done in a framework of standardized assays that identify correlates for protection, then the number of antigens to go through to GMP products will be greatly increased. A milestone towards achieving this was the sequencing of several malarial genomes. The completion of the *P. falciparum* genome in 2002[210] provided researchers with the opportunity to develop these approaches through a better understanding of malarial parasite biology and its interaction with its hosts. Approximately 5300 *P. falciparum* genes were revealed upon completion of the genome and nearly 65% of these were hypothetical genes of unknown function. There is a real need for novel methods of high throughput characterization in order to rapidly interrogate these hypothetical genes to discover their usefulness as novel putative vaccine candidates. Hall et al.[211] recently completed 'a comprehensive survey of the Plasmodium life cycle' (reviewed in Fraunholz[212]). This study defined 4391 genes in *P. falciparum* with homologs in rodent malaria parasites allowing these species (which are more amenable to genetic manipulation)[213] to be used in determining gene function. This integration of genomic, transcriptomic, and proteomic datasets offers the prospect of a rationale approach to identifying key vaccine and drug targets using high throughput methodologies. Such an approach will be more powerful when used in conjunction with classical biochemical and cell biology techniques.[212]

The development of transfection technologies for *P. falciparum* (including the recent advances in double crossover integration into the genome)[214] provides a powerful molecular tool to define the role of individual genes within the cellular biology of this organism. However, the application of this technique is limited by the process being relatively inefficient, labor intensive, and time consuming. Genetic manipulations of essential asexually ex-

pressed genes are often not possible due to deleterious effects. Despite these limitations, gene knockout has been effectively used to characterize genes involved in processes such as the invasion of red blood cells[215] and cellular adhesion,[216-218] many of which represent either current or putative vaccine candidates.

The ability to identify homologs in rodent malaria systems means that the more efficient and rapid knockout technologies of *P. Berghei* can be used to characterize gene function in *P. falciparum.*[213] Alternatively, more rapid gene knockout technologies for *P. falciparum* using transposable elements[219] will speed up gene characterization; Balu and colleagues[219] employed the lepidoptearan transposable element *piggyBac* to efficiently transform *P. falciparum*. Such a technique offers the potential to perform large scale mutagenesis of the *P. falciparum* genome in order to screen for interesting phenotypes and identify the loci which have been disrupted. Other advances in transfection techniques include the use of the mycobacterium Bxb1 integrase to produce genetically and phenotypically homogeneous transgenic parasite populations.[220] This integration occurred within 1 month in the absence of drug pressure.[221] With regards to the knockout of essential genes, the development of a successful inducible promoter (as has been achieved in the apicomplexan *Toxoplasma gondii* and currently being developed in *P. falciparum*)[222] remains a key goal. Such a system would allow essential genes to be knocked out and their function studied by regulating the expression level of episomal copies as well as looking at the effects of inappropriate timing of expression. In addition to gene knockout, green fluorescent protein (GFP) tagging has provided the ability to look at the cellular location of putative vaccine candidates in live cells, allowing scientists to investigate processes such as proteolytic cleavage and cellular export.

10. Conclusion

In the last 5 years, malaria vaccine development has seen advances on many fronts. A long-lasting immunity with a significant protective effect generated by RTS,S/AS02A has proved that a vaccine is technically feasible. This is coupled with a dramatic increase in the number of candidates being taken forward into GMP development for human trials and development on vaccine delivery platforms. The development of RTS,S/AS02A has undoubtedly been advanced due to an agreement between GSK Biologicals and the MVI. The MVI is a nonprofit organization dedicated to overcoming the barriers to malaria vaccine development. CSP has been under development as a vaccine for 18 years by GSK Biologicals, with a current cumulative expenditure of between $US75 and $US100 million. The financial and political power behind MVI, coupled with the definition of strategic goals to overcome the barriers to vaccine development and licensing is key to the scale of undertaking necessary to move an increasing number of vaccine candidates through the pipeline. In addition to MVI, the European Malaria Vaccine Initiative, the US Army Medical Research and Materiel Command (via the WRAIR), as well as private pharmaceutical companies such as GSK Biologicals are providing key resources and technical knowledge to push forward vaccine development.

Although there is a real philanthropic nature to the development of malaria vaccines for developing countries, there is realization that an increase in GDP resulting from the distribution of an efficacious vaccine[223] would produce a substantial increase in market potential for European and American countries. Therefore, there is real financial incentive for private companies to become involved in the race for a malaria vaccine. Technological progress in the field of vaccine development to protect against organisms such as group A *Streptococcus* can also be applied to the development of malaria vaccines. The ability of chimeric constructs to deliver strain-transcending immunity make it reasonable to predict that multiple allelic forms and/or multiple life-cycle stages of *P. falciparum* could be targeted by a malaria vaccine to increase efficacy. Promising results have also been achieved in genetically attenuating *P. falciparum* for the production of live attenuated vaccines, an approach which is receiving serious financial investment.

These positives must be tempered with some concerns. So far, the best efficacy seen with a malaria vaccine has been around 30% against clinical malaria, yet currently there is no vaccine widely in use as a public health tool that does not provide at least 75% sustained protection against infection and/or disease. It is also worth noting that all of the vaccines widely used as a public health tool in infants and young children in the developing world were first licensed, manufactured, and sold for use in children and/or adults in the developed world. While there are attempts to define better preclincial and clinical models to predict the efficacy of vaccines, there are no current models to accurately predict the usefulness of an asexual protein as a vaccine candidate. In addition, there are still no reliable immunologic correlates of protection for natural infections or immunizations. The requirement for multiple allelic forms to overcome allelic diversity (although technically possible) will no doubt increase the complexity of a vaccine and its developmental and production costs. This would also be the likely outcome of combining multiple antigens to target different life-cycle stages of the parasite. It can be seen that the field of malaria vaccine research has several challenges to overcome before a viable product for global usage can be produced.

Whether the increase in GDP of developing countries and the philanthropy of developed countries could sustain annual costs of

around $US1 billion to buy and deploy an efficacious malaria vaccine is unknown. Were a licensed malaria vaccine to become a reality, and a successful vaccination program begin to reduce malaria-associated morbidity/mortality, then there is a real prospect that the lifespan of an individual malaria vaccine would be limited. The ability of the malaria parasite (and its vectors) to overcome successful intervention programs via a process of mutation and gene flow has been a recurrent theme in public health programs. Even if a vaccine is successfully developed and deployed, perhaps the biggest challenge for researchers and public health specialists will be predicting and responding to future evolutionary changes in this parasite that could render such a vaccine ineffective.

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