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Towards an RTS,S-Based, Multi-Stage, Multi-Antigen Vaccine Against Falciparum Malaria: Progress at the Walter Reed Army Institute of Research

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Towards an RTS,S-based, multi-stage, multi-antigen vaccine against falciparum malaria: progress at the Walter Reed Army Institute of Research☆

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Abstract

The goal of the Malaria Vaccine Program at the Walter Reed Army Institute of Research (WRAIR) is to develop a licensed multi-antigen, multi-stage vaccine against *Plasmodium falciparum* able to prevent all symptomatic manifestations of malaria by preventing parasitemia. A secondary goal is to limit disease in vaccinees that do develop malaria. Malaria prevention will be achieved by inducing humoral and cellular immunity against the pre-erythrocytic circumsporozoite protein (CSP) and the liver stage antigen-1 (LSA-1). The strategy to limit disease will target immune responses against one or more blood stage antigens, merozoite surface protein-1 (MSP-1) and apical merozoite antigen-1 (AMA-1). The induction of T- and B-cell memory to achieve a sustained vaccine response may additionally require immunization with an adenovirus vector such as adenovirus serotype 35. RTS,S, a CSP-derived antigen developed by GlaxoSmithKline Biologicals in collaboration with the Walter Reed Army Institute of Research over the past 17 years, is the cornerstone of our program. RTS,S formulated in AS02A (a GSK proprietary formulation) is the only vaccine candidate shown in field trials to prevent malaria and, in one instance, to limit disease severity. Our vaccine development plan requires proof of an individual antigen's efficacy in a Phase 2 laboratory challenge or field trial prior to its integration into an RTS,S-based, multi-antigen vaccine. Progress has been accelerated through extensive partnerships with industrial,

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academic, governmental, and non-governmental organizations. Recent safety, immunogenicity, and efficacy trials in the US and Africa are presented, as well as plans for the development of a multi-antigen vaccine.
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1. Introduction

The Walter Reed Army Institute of Research (WRAIR) in Silver Spring, Maryland is dedicated to developing drugs, diagnostic devices, and vaccines to protect military personnel against disease, particularly tropical diseases such as malaria [1]. Military medical research has led to the development and field-testing of anti-malarial drugs including mefloquine, primaquine, doxycycline, atovaquone-proguanil (Malarone), tafenoquine, and halofantrine that are active against drug-resistant strains of *Plasmodium falciparum* [2]. These anti-malarial drugs have demonstrated substantial public health benefit. Despite the availability of modern antimalarial drugs, mosquito-repellants, bed nets and other countermeasures, malaria threatens the success of modern militaries, most recently Australian and Thai forces in East Timor (2000), British troops in Sierra Leone (2001) and the US Marines in Liberia (2003). The US military requires a safe, well-tolerated highly effective vaccine that prevents malaria or very significantly limits the severity of disease in vaccinees who develop malaria, and confers sustained protection. Endemic populations suffer some 3 million deaths and 500 million episodes of this dread disease annually [3]. A vaccine with this profile may also be of potential public health benefit to these populations. Recent excellent reviews broadly address the status of current malaria vaccine efforts, considerations in vaccine design, and the formidable obstacles to vaccine development [4–6]. This brief article focuses on the successful WRAIR-GSK partnership that led to the RTS,S-based malaria vaccine candidates, the results and implications of recent RTS,S/AS02A clinical and field trials, and then outlines plans to develop an RTS,S-based multi-antigen, multi-stage malaria vaccine that would fulfill the requirements of the US military.

2. Development of RTS,S

2.1. Initial circumsporozoite protein vaccines

The gene coding for the circumsporozoite protein (CSP) was the first *P. falciparum* gene to be identified and cloned [7]. The CS proteins of all malaria species contain a central segment of species-specific amino acid repeat sequences flanked by non-repeat regions [8,9]. In the case of *P. falciparum*, the central portion contains approximately 40 repeats of the amino acid sequence asparagin–alanine–asparagines–proline (NANP) and several asparagines–valine–aspartic acid–proline (NVDP) repeats [10]. The central repeating region contains immunodominant B-cell epitope(s) [11], whereas the majority of epitopes responsible for the induction of CD4 and CD8 T-cells are located within the flanking regions. Two alum-adjuvanted candidate vaccines based on the CSP repeat sequences were developed in the mid 1980s [12], tested in humans at the University of Maryland [13] and at WRAIR [14], and found to be safe; however, both suffered from generally poor immunogenicity. In most volunteers, repeated immunization induced relatively low titers of antibodies against the repeat region of the CSP. Nevertheless, a small number of volunteers who did respond well were protected against homologous experimental sporozoite challenge. These encouraging challenge data served as a proof of concept that antibody-mediated sterile immunity against malaria could be induced in humans by immunization with a recombinant or synthetic pre-erythrocytic stage subunit vaccine. In these studies with a limited number of volunteers, protection seemed to be associated with high antibody levels; therefore, it was assumed that the development of more immunogenic versions of these vaccines would lead to better efficacy. Subsequent sporozoite vaccines, also based on the NANP repeats, contained modifications conducive to the enhancement of humoral immune responses, such as conjugation of recombinant CSP repeat region with *Pseudomonas aeruginosa* exotoxin A (R32ToxA)[15] and formulation of a similar antigen (R32NS1) in liposomes containing monophosphoryl lipid A (MPL)[16] or an emulsion of MPL, mycobacterial cell wall skeleton, and squalene (Detox®, Ribi Immunochem) [17,18]. Each of these strategies resulted in generally higher antibody levels, but surprisingly did not significantly enhance efficacy upon live sporozoite challenge [19–21], thus suggesting that antibody responses to NANP repeats were not alone sufficient to induce significant protective efficacy.

2.2. WRAIR-GSK development of RTS,S

While these clinical studies were in progress, the role of protective T-cell responses directed against the liver-stage form of the parasite was elucidated in greater detail. Studies in rodents and humans immunized with irradiated sporozoites revealed that not only antibodies, but also CD4 and CD8 cells that exhibited cytotoxic T-cell (CTL) responses or produced cytokines such as interferon-γ were important components of the protective immune network against the pre-erythrocytic stage of *P. falciparum* [22]. Target epitopes for these T-cells have been localized to the C-terminal region of the CSP [23–26]. The recognition that peptide sequences lying outside the CSP repeat were important for induction of humoral and cellular responses against the hepatic stage led to the inclusion of non-repeat

portions of the molecule in the next generation of CSP-based vaccines.

In 1984, GlaxoSmithKline in collaboration with WRAIR pioneered the use of the hepatitis B surface antigen (HBsAg) as a carrier matrix for the central repeat region of the *P. falciparum* CSP [27]. The initial construct, referred to as R16-HBsAg, was formulated with alum; it proved safe and immunogenic in 20 hepatitis B-seronegative adults at the University of Nijmegen [28]. In 1987, following the recognition of the potential role of cell-mediated immunity and of non-repeat sequence of CSP, a new CSP–HBsAg fusion protein was developed at GSK, which incorporated the CSP C-terminal flanking region known to contain B- and T-cell epitopes into a chimeric gene expressed in *Saccharomyces cerevisiae*. This construct was named "RTS" to indicate the presence of the CSP repeat region (R), T-cell epitopes (T), and the hepatitis B surface antigen (S). Exigencies of purification led to the co-expression and purification of additional antigen in a ratio of RTS to S of 1:4, hence the name "RTS,S." A process compatible with GMP manufacturing of RTS,S was then developed at GSK.

2.3. First trials of RTS,S

The first clinical trial of RTS,S evaluated two formulations developed at GSK for safety and efficacy in malaria naïve adults in 1992 at the Walter Reed Army Institute of Research, The formulations were RTS,S and alum ($N = 10$), and RTS,S with monophosphoryl lipid A and alum ($N = 10$). Both vaccines proved to be safe and well tolerated. RTS,S/MPL/alum elicited higher antibody responses against the CSP repeat and C-terminal region than did RTS,S/alum. After sporozoite challenge, zero of six RTS,S/alum vaccinees and two of eight RTS,S/MPL/alum vaccinees were protected [29]. These encouraging results were considered to warrant further improvement of the vaccine. Consequently, GSK and WRAIR initiated a rigorous comparative preclinical safety and immunogenicity evaluation of six GSK proprietary formulations of RTS,S in rhesus monkeys at the Armed Forces Research Institute of Medical Sciences to improve upon both the humoral and cell-mediated immunogenicity of RTS,S/MPL/alum. One formulation, consisting of RTS,S formulated in an oil-in-water emulsion with MPL and QS21, demonstrated superior antibody and delayed type hypersensitivity responses, and is today designated RTS,S/AS02A [30].

2.4. First trials of RTS,S/AS02

In 1996, volunteers immunized with either RTS,S/AS03 (an oil-in-water emulsion without additional adjuvants) or RTS,S/AS02A in a Phase 2A trial at WRAIR developed the highest anti-repeat antibody responses ever induced by a CS-based vaccine, but RTS,S/AS02A induced a higher T-cell response as measured by IFN-γ production in an ELISPOT assay [31]. Following laboratory-based sporozoite challenge, the RTS,S/AS03 formulation was marginally protective (two of seven); in contrast, RTS,S/AS02A protected an unprecedented six of seven volunteers [32]. A single determination of the duration of protection 6 months after initial challenge found that only one of five previously protected RTS,S/AS02A-immunized volunteers was still protected [33]. Preliminary dose optimization studies at WRAIR have since confirmed that two or three doses of RTS,S/AS02A consistently confer significant protection (sterile immunity) and significant delays in the prepatent period in RTS,S/AS02A vaccinees who develop malaria as compared to concurrent infectivity controls [34].

2.5. Lyophilized RTS,S

After stability testing revealed a limited shelf life of RTS,S in the AS02A adjuvant, RTS,S was reformulated at GSK as a stable frozen lyophilized cake and filled in single dose vials for reconstitution with AS02A just prior to injection. A comparative trial at WRAIR demonstrated the equivalent safety and immunogenicity of the liquid and lyophilized preparations. Further, lyophilized RTS,S formulated with AS02A provided equivalent protection against experimental malaria challenge as well as the earlier liquid RTS,S/AS02A formulation (Kester, unpublished results). Subsequent trials of RTS,S have all been performed with the lyophilized version.

2.6. Accelerated RTS,S/AS02A schedules

During initial trials, the RTS,S/AS02A vaccine was administered on a 0-, 1- and 6–9-months schedule. Subsequently, a Phase 2A trial at WRAIR compared the safety, immunogenicity and efficacy of lyophilized RTS,S, formulated with AS02A on two different accelerated schedules. Forty volunteers received a full dose of RTS,S/AS02A on either a 0-, 1- and 3-months schedule ($N = 20$) or on a 0-, 7-, and 28-days schedule ($N = 20$). Following standard sporozoite challenge, 45 and 38%, respectively, of vaccinees were completely protected against malaria and there was no increase in reactogenicity versus historical controls (Kester, unpublished results). These data provided support for subsequent accelerated schedules of evaluation for RTS,S/AS02A in Africa and for other candidate antigens adjuvanted with AS02A at WRAIR.

2.7. RTS,S/AS02A field trials

The initial trial of RTS,S/AS02A, conducted by the Medical Research Council (MRC) in The Gambia in healthy adult males, demonstrated unprecedented effects, namely a 34% reduction in instance of first parasitemia (95% CI 8–53%; $p = 0.014$) over a 16-weeks efficacy period [35,36], and efficacy appeared not to be strain specific [37]. Subsequently, GSK and the Malaria Vaccine Initiative at PATH (MVI)

entered into a partnership that has sponsored the initiation of pediatric clinical development of RTS,S/AS02A. In initial pediatric studies conducted by the MRC in The Gambia and CISM in Mozambique, the vaccine was shown to be safe and highly immunogenic in children 1–11 years of age (Bojang et al., submitted for publication; Macete et al., submitted for publication). In the recently reported Phase 2B efficacy study conducted by CISM and the University of Barcelona in Mozambique, it was reported that over a 6-months period in children 1–4 years of age, RTS,S/AS02A conferred 30% (95% CI 12–45%; $p = 0.004$) efficacy for first clinical episodes and 58% (95% CI 16–81%; $p = 0.019$) efficacy for severe malaria due to *P. falciparum* [38]. Longitudinal follow-up is in progress to define the long-term safety and the magnitude and duration of RTS,S/AS02A's benefit in young children, and studies are planned to develop an RTS,S vaccine for infants in conjunction with the Expanded Program on Immunisation [39].

3. Potential RTS,S improvements

Until the correlates of vaccine-induced protective immunity are known, Phase 2A challenge and Phase 2B field trials will be required to confirm the efficacy of particular improvement strategies. Decision points for integrating improvements are listed in Table 1.

3.1. RTS,S efficacy

Efficacy estimates for Phase 2A trials of RTS,S/AS02A based only on the occurrence of parasitemia overlook the important biologic effect represented by the consistent prolongation of the prepatent period in RTS,S/AS02A vaccinees. In recent trials, approximately 40% of adult volunteers immunized with two or three doses of RTS,S/AS02A experience complete protection and an additional 40–45% note a roughly 2 days delay in the pre-patent period when compared to simultaneous infectivity controls. This effect is attributable to either a reduction of viable sporozoites or to an interruption of hepatic schizogony or both resulting in a significant reduction in the release of hepatic merozoites [34,40]. Encouraged by these results, we have embarked on parallel development efforts to augment RTS,S/AS02A efficacy.

3.2. RTS,S and an adenovirus vector

In 1997, scientists at New York University's (NYU) School of Medicine first reported in a series of publications that a recombinant adenovirus expressing the CSP generated sustained protection in mice against lethal challenge with *P. yoelii* sporozoites by a variety of immune mechanisms [41–43]. Later, in a joint effort between NYU and Crucell, a biotechnology company in the Netherlands, it was shown that a novel recombinant adenovirus 35 vector expressing CSP also protected mice against *P. yoelli* (Goudsmit, unpublished). The recombinant adenovirus 35 is a non-replicating viral vector chosen for clinical vaccine development because there is minimal worldwide seroprevalence of neutralizing antibody that would interfere with its use as a vaccine [44–47]. Recently, a collaboration between Crucell, GSK, and WRAIR was established for the purpose of testing an adenovirus 35 (Ad 35) CSP vaccine directed against *P. falciparum*, designated Ad 35 CS as well as the combination of Ad 35 CS and RTS,S in a so called "prime/boost" regimen. Thus, in 2003 preclinical evaluations began of Ad 35 CS alone and in combination with RTS,S in the standard rhesus monkey safety and immunogenicity model at WRAIR. Contingent upon the results of this study, Phase 2A clinical trials are planned for 2006, with the support of the National Institute of Allergy and Infectious Diseases.

3.3. RTS,S with additional antigens

We expect that the efficacy of an RTS,S-based malaria vaccine could be improved by combination with one or more protective antigens. Our vaccine development strategy is first to establish a protective effect of additional protein antigens prior to co-formulating them with RTS,S as a combined, multi-antigen vaccine.

3.3.1. Merozoite surface protein-1 (MSP-1)

Merozoite surface protein-1, the first antigen discovered on the surface of erythrocytic stage merozoites, is also expressed in all generations of merozoites, including those released at the end of hepatic schizogony [48]. The C-terminal portion of the MSP-1 is the target of protective immunity as suggested by epidemiological studies [49,50] and as demonstrated by the protection of *Aotus* monkeys against malaria challenge by immunization with 42-kDa C-terminal MSP-1

Table 1
Scheduled evaluation of potential RTS,S improvements

Technology	Rationale	Example	Criteria and location	Decision date
Antigen	Target hepatic stage parasites	Liver stage antigen-1 (LSA-1)	Phase 2A at WRAIR	2006
Antigen	Target blood stage	Merozoite surface protein-1 (MSP-1)	Phase 2B in Kenya	2006
Antigen	Target blood stage	Apical merozoite antigen-1 (AMA-1)	Phase 2B in Mali	2007
Vector	Increase magnitude and duration of CMI	Adeno 35/full length CSP (Ad 35 CS)	Phase 2A at WRAIR	2007

CMI: cell-mediated immunity; Phase 2A: preliminary vaccine efficacy trial against experimental malaria challenge; Phase 2B: preliminary vaccine efficacy trial in malaria endemic country; CSP: circumsporozoite protein; and Adeno 35: adenovirus serotype 35.

recombinant protein [51,52]. In the late 1990s, with sustained support from the USAID and in the context of the GSK/WRAIR collaborations, a 42-kDa C-terminal MSP-1 protein based on the *P. falciparum* 3D7 clone was manufactured to GMP standards at the WRAIR [53]. In preclinical [54] and Phase 1 trials at WRAIR, MSP-1 formulated with AS02A proved to be safe and highly immunogenic, eliciting high titer antibodies as measured by ELISA and by immunofluorescence assay against parasitized red blood cells, as well as T-cell responses characterized by IFN-γ production. The volunteers also produced functional antibody as measured by an in vitro growth inhibition assay (Ockenhouse, unpublished). A subsequent Phase 2A trial at WRAIR tested MSP-1/AS02A alone and in combination with RTS,S/AS02A. MSP-1/AS02A alone did not delay parasitemia, and in combination with RTS,S/AS02A it did not improve upon or interfere with the protective efficacy of RTS,S/AS02A. The trial design required treating volunteers as soon as they developed a detectable parasitemia, and thus the data did not allow determination as to whether or not MSP-1/AS02A would limit parasitemia and disease (Cummings, unpublished). Next, the MVI, USAID, GSK, WRAIR and the Kenya Medical Research Institute (KEMRI) conducted Phase 1 trials of the MSP-1/AS02A in adults and in children in malaria endemic Western Kenya from 2002 to 2004. In parallel, and with the additional support of the NIAID, University of Bamako investigators completed an additional Phase 1 safety and immunogenicity trial of MSP-1/AS02A in healthy adults in Bandiagara, Mali in 2004. On the basis of the favorable safety and immunogenicity data for MSP-1/AS02A, an independent Data Safety Monitoring Board recommended proceeding to a Phase 2B trial in children ages 1–4 years. This trial will commence in 2005, with support from MVI, USAID, and GSK, and will evaluate vaccine efficacy against clinical disease in children in Western Kenya.

3.3.2. Apical merozoite antigen-1 (AMA-1)

Apical merozoite antigen-1 (AMA-1), originally identified as a blood stage protein, is an essential antigen [55] expressed in sporozoite [56], late stage hepatic parasites [57], and in erythrocytic merozoites. AMA-1, like MSP-1, is the target of protective immunity, as supported by epidemiological observations [58], in vitro demonstrations [55], and by protection studies in which mice [59] or monkeys [60] immunized with recombinant forms of AMA-1 were protected from lethal challenge with homologous parasites. Scientists at the WRAIR have produced a correctly folded GMP recombinant protein in *E. coli* based upon the *P. falciparum* 3D7 strain AMA-1 ectodomain [61]. This antigen was vialed as a single-dose lyophilized cake intended for formulation with AS02A just prior to administration. In the context of the GSK/WRAIR collaboration, a Phase 1A dose-escalating trial was conducted at WRAIR's Clinical Trials Center in 2003. Twenty-three healthy adult volunteers received three intramuscular doses of AMA-1 formulated with AS02A adjuvant on a 0-, 1-, and 2-months schedule. AMA-1/AS02A proved safe and immunogenic. Volunteers developed parasite-specific antibody by IFA, functional antibody as measured by an in vitro growth inhibition assay, and cellular responses as measured by lymphoproliferative and IFN-γ responses by ELISPOT assays (Polhemus, unpublished). Two additional trials of AMA-1/AS02A are underway. A Phase 1B controlled trial in Bandiagara, Mali, supported by the National Institute of Allergy and Infectious Diseases and led by investigators from the University of Bamako, is underway to evaluate two different doses of AMA-1/AS02A in healthy adults on a 0-, 1-, and 2-months schedule. A Phase 2B trial of AMA-1/AS02A in children at risk of disease with the primary endpoint of clinical disease is planned for 2006 in West Africa.

3.3.3. Liver stage antigen-1 (LSA-1)

Liver stage antigen-1 was identified as a potential vaccine antigen in 1990 [62]. There are no accepted homologues of this *P. falciparum* antigen in murine or simian plasmodia. Therefore, the case for LSA-1 as a vaccine candidate rests upon strong epidemiological correlations of immune responses with clinical immunity to malaria in malaria-endemic populations, and with the correlation of acquired LSA-1 immune responses with protection in irradiated sporozoite immunization studies [63]. WRAIR scientists have produced *E. coli*-expressed recombinant *P. falciparum* LSA-1 protein under GMP conditions as a candidate malaria vaccine. In 2004, WRAIR partnered with GSK and PATH's Malaria Vaccine Initiative to develop LSA-1 as a vaccine [64]. Phase 2A trials are planned for late 2005/early 2006 at WRAIR's Clinical Trials Center that will evaluate the safety, immunogenicity and efficacy of LSA-1.

4. Conclusion

RTS,S/AS02A, the candidate malaria vaccine in the most advanced stage of development, is the result of a sustained 17-years effort by partners committed to the development of a malaria vaccine. The immunologic correlates of RTS,S-induced protection have not been identified yet but trends have begun to emerge, allowing more rational development to supplant empiricism, and to enable a greater focus of resources on the most promising potential improvements of RTS,S. The recent report that RTS,S/AS02A had a significant positive impact on clinical and severe malaria in children in Mozambique strongly support our working hypothesis that a more effective RTS,S-based vaccine could be developed that would better meet the US Army military needs and perhaps also benefit global public health needs.

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Potential conflicts of interest statement: Christian Darko, Lalitha Nair, Lisa Ware, Collette Hillier, Jeffrey Lyon,

Evelina Angov, David Lanar and Sheetij Dutta hold patents for the production and formulation of one or more discussed protein vaccine candidates (i.e. MSP-1, LSA-1, and AMA-1). Drs. Ripley Ballou and Joe Cohen are employees of GlaxoSmithKline Biologicals, the manufacturer of RTS,S and of the discussed proprietary adjuvant AS02A. Joe Cohen and Nathalie Garçon are listed as inventors of patented malaria vaccine and adjuvant formulations; however neither individual hold the patent for a malaria vaccine or for adjuvant formulations. Drs. Maria Pau and Jaap Goudsmit are employees of Crucell, the manufacturer of the described adenovirus vector proposed for a malaria vaccine. Drs. Stewart and Heppner have filed patent applications for the use of vaccines described in this article.

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