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Malaria vaccines

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1. ABSTRACT

More than 120 years after Alphonse Laveran's discovery of the blood-stage malaria parasite, there is no licensed malaria vaccine and malaria remains the world's most serious parasitic disease. Efforts to develop a vaccine have been thwarted by the complexity of the parasite's life cycle and the ability of the parasite to suppress and evade the immune response. Currently, there are several candidate vaccines in clinical trials and many more candidate vaccines that have shown efficacy in animal models or are based on studies of the immune responses of people who are resistant to malaria. The sequencing of the genomes of *Plasmodium falciparum* and *Plasmodium yoelii*

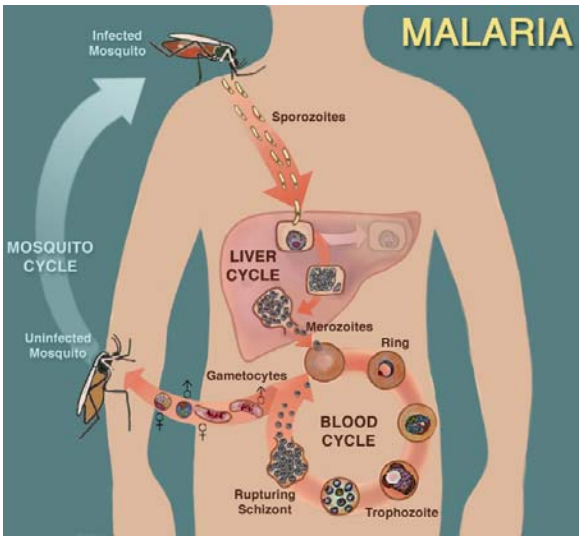

Figure 1. Life cycle of malarial parasites.

yoelii in 2002 is expected to result in the identification of previously-unknown candidate vaccine targets from various stages of the Plasmodium life cycle. A great deal of effort is going into identifying the correlates of protection, potentially allowing more efficient testing of candidate vaccines in the future. The fact that a vaccine candidate has shown partial protection in field trials is a reason for hope that, with the proper effort and support, effective vaccines against malaria can be developed.

2. INTRODUCTION

Malaria causes an estimated 1-3 million deaths a year (1,2). The global malaria crisis is worsening, particularly in sub-Saharan Africa (3). Current efforts to control malaria employ three strategies: control of the mosquito vectors, development and use of antimalarial drugs for prophylaxis and treatment, and development of vaccines. Improvements can and should be made in all these areas, but because of the ability of the parasite to develop resistance to new drugs and the ability of the mosquito vectors to develop resistance to insecticides, vaccines against malaria are urgently needed.

There are several difficulties in developing anti-malarial vaccines: 1) Natural immunity to malaria after infection is not complete and appears to require constant restimulation by the parasite. Therefore, there is only an imperfect natural model of immunity to follow in designing a vaccine. 2) Malarial parasites evade and suppress the immune response. 3) Some of the same immune mechanisms that kill the parasite can cause illness in the host, depending on factors such as the timing and intensity of the response. 4) The vast majority of malaria cases occur in poor countries with very small budgets for public health and research. 5) Genes for Plasmodium proteins are difficult to express at high yield and with proper folding in commonly-used expression systems. 6) Existing aluminum-containing adjuvants have generally been insufficiently potent for malaria vaccines, requiring the development of new adjuvants (4).

Four of the over 100 species of *Plasmodium* account for virtually all malaria infections in humans: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, and Plasmodium malariae*. The majority of cases and almost all of the deaths are due to *P. falciparum*. Disease caused by *P. ovale* and *P. malariae* is relatively rare (5).

This review will focus mainly on subunit vaccines to Plasmodium falciparum. Only a few of the many vaccines being tested will be discussed. More complete reviews of the various vaccines in late preclinical and clinical development are Ballou and coworkers (6) and Ballou (4). The World Health Organization maintains a list of more than 90 malaria vaccine candidates at various stages of development at www.who.int/vaccine_research/documents/en/malaria_table.pdf.

3. LIFE CYCLE OF MALARIA PARASITES

The life cycle, morphology, metabolism, genetics, and culture of *Plasmodia* have been reviewed by Sinden and Gilles (7). A proteomic view of the life cycle of *P. falciparum* is given by Florens and coworkers (8). Figure 1 is a diagram of the life cycle of *Plasmodium falciparum*. When a female *Anopheles* mosquito takes a blood meal, a median number of perhaps 15-25 sporozoites are deposited in the dermis of the host (9,10). Some of the sporozoites are drained by lymphatic vessels and stop at proximal lymph nodes, (11) while others enter blood capillaries and rapidly make their way to the liver, where they migrate through resident macrophages (Kupffer cells) and infect hepatocytes. Inside the hepatocytes, the sporozoites undergo many nuclear divisions to form schizonts, each of which ultimately gives rise to more than 10,000 merozoites. The merozoites then infect red blood cells, where they undergo a cycle of reproduction and reinfection that causes most of the symptoms of malaria. When the extracellular merozoite invades a red blood cell, it becomes round due to the degradation of the inner membrane complex and subpellicular microtubules, and transforms into a trophozoite. The trophozoite (from the Greek *trophē*, nutrition) ingests host cell cytoplasm. Following this stage is schizogony, in which there is nuclear division, formation of organelles, and formation of membranes that segment the cytoplasm to form a separate mass around each nucleus. The resulting schizonts rupture, each one releasing some 6-32 new merozoites, which go on to infect new red cells and begin the cycle again. The lysis of the red cells (along with bone marrow suppression) is one of the causes of the anemia that is associated with malaria (1). Instead of reproducing asexually, an unknown factor causes some of the parasites in the blood to develop into male and female gametocytes. Some of these gametocytes are taken up by a mosquito in another blood meal. The gametocytes differentiate into gametes and emerge from the erythrocytes. Fertilization occurs in the mosquito midgut to produce motile zygotes, which differentiate into motile ookinetes. The ookinetes penetrate the gut wall and differentiate into mature oocysts. Meiosis takes place in each oocyst to produce thousands of haploid sporozoites, which are released into the mosquito body cavity (hemocoel) and migrate to the salivary glands of the

mosquito, where they can be injected into a human host along with anticoagulant-containing saliva during a blood meal.

There are several possible strategies for making a malaria vaccine: 1) Pre-erythrocytic vaccines are designed to elicit an immune response that inactivates or kills sporozoites, prevents them from infecting hepatocytes, or kills infected hepatocytes or the parasites within infected hepatocytes. 2) Blood stage vaccines target free merozoites or the invasion of red blood cells by merozoites. 3) Vaccines may be designed to prevent or to counteract the pathological responses of the host to the parasite. For example, antibodies may be elicited to the glycosylphosphatidylinositol molecule that appears to elicit the production of inflammatory mediators. 4) Transmission-blocking vaccines can be made to inhibit the growth or fertilization of the sexual stages of the parasites. These vaccines would not protect the person who is vaccinated but would protect other people in the same geographic area, because mosquitoes would not contain infectious sporozoites. Because the immune response to malaria parasites tends to be stage-specific, and because the parasite undergoes a variety of genetic changes to evade the immune response, it is believed that it may be necessary to combine antigens from different life cycle stages to develop a highly-effective malaria vaccine.

4. INNATE AND NATURALLY-ACQUIRED IMMUNITY

4.1. Innate response

The first step in the innate immune response is the activation of dendritic cells (12). The innate immune response to *Plasmodium* infection, which includes dendritic cells, natural killer cells, monocytes, macrophages, and gamma-delta T cells, has been reviewed by Stevenson and Riley (13) and Urban and coworkers (14). Pichyangkul and coworkers (15) have found that blood stage parasites activate murine dendritic cells and human plasmacytoid dendritic cells via Toll-like receptor 9 (TLR9). The activation of dendritic cells is important for both the innate and the adaptive immune responses. The activation of natural killer (NK) cells by dendritic cells is potentially a critical event during the innate immune response to malaria because NK cells are one of the main producers of interferon gamma, which is believed to contribute to protection against blood stage malaria (16) and is also important in protection against liver-stage malaria (17). In the innate response to human malaria, high-level interferon gamma responses are associated both with control of parasite growth and with clinical symptoms such as fever or febrile chills (18). The role of interferon gamma in adaptive responses is discussed in the sections on clinical trials for pre-erythrocytic and blood-stage vaccines, sections 6.1 and 7.1 respectively. The activation of NK cells is dependent on contact with infected red blood cells, and IL-12 and to a certain extent IL-18 are critical for NK cell activation and interferon-gamma production (14). Activated NK cells can kill *P. falciparum*-infected red blood cells (14). Baratin *et al.* (19) found that NK and macrophage cooperation, which includes the production of IL-18 by macrophages, is essential in the innate responses of NK cells to *P. falciparum*. Phagocytosis of parasitized red blood cells is another important non-specific immune defense mechanism (reviewed in 14). Binding to CD36 expressed on monocytes and macrophages mediates non-opsonic phagocytosis of parasite-infected red blood cells (14). In addition, gamma-delta T cells are rapidly activated by live infected red blood cells and can inhibit the growth of asexual blood stages *in vitro* in a contact-dependent interaction (20).

4.2. Adaptive response

The adaptive immune response to malaria has been reviewed by Engwerda and Good (21). Exposure to malaria rarely if ever results in sterile immunity. However, in areas of high to moderate rates of transmission, severe and fatal malaria is mostly restricted to children, and adults suffer from no or mild morbidity (22, 23). Pregnant women, especially primigravidae, have an increased risk of premature delivery, increased perinatal mortality, and reduced birth weight of the newborn, because of sequestration of infected erythrocytes in the placenta (24; reviewed in 25). Repeated reinfection is necessary to retain immunity to clinical symptoms (reviewed in 26). In highly endemic areas, intermittent parasitemia is almost universal (26), but many individuals with parasitemia show no symptoms of disease. Up to 80% of immune people with parasitemia may be completely asymptomatic (27, 28). The immunity associated with latent infection has been labeled "premunition" (reviewed in 29).

The mechanisms involved in adaptive immunity to malaria are believed to vary, depending on the stage of the parasite life cycle that is being targeted. These mechanisms are described below in section 6.1, Clinical Trials of Pre-erythrocytic vaccines, Section 6.3, Liver-stage vaccines, and Section 7.1, Clinical Trials of blood-stage vaccines.

5. ANIMAL MODELS

Rodent models for malaria have been reviewed by Landau and Gautret (30) and Langhorne *et al.* (31), and primate models have been reviewed by Gysin (32).

The most commonly used animal models for malaria vaccine research are the mouse and the *Aotus* monkey. Mice are infected by *Plasmodium berghei, P. yoelii, P. chabaudi*, and P. *vincke*i, and the *Aotus* monkey can be infected by *P. falciparum* and *P. vivax*. *Plasmodium knowlesi* is rapidly fatal in the rhesus monkey (33). *Plasmodium vivax* has been adapted to the new world monkey *Aotus nancymai (*34*)*, and this model is useful for preclinical drug and vaccine development (35, 36, 37). However, the predictive value of the *Aotus* model for vaccine efficacy in humans has not been evaluated, and the fact that the host-parasite relationship is abnormal has to be borne in mind. (35). *P. falciparum* can cause a lethal infection in *Aotus vociferans* (38). Aside from humans, only chimpanzees are susceptible to the liver-stage infection with *P. falciparum* that causes malaria (39).

There is disagreement as to whether new world monkeys should be on the critical path for malaria vaccine development. For vaccine development, the phrase "critical path" refers to the essential tasks required for a selected antigen to proceed to phase 3 clinical trials in support of licensure. There is insufficient space in the present article to do justice to the arguments made on both sides; the interested reader is referred to the original papers. Briefly, Stowers and Miller (40) say that challenge trials in New World monkeys are the most efficient way to compare an immunogen made in different expression systems and by different refolding or purification methods. They point out that surrogate endpoints are used for human trials of blood-stage vaccines and claim that monkey efficacy trials would be more relevant and allow a choice of immunogen before expensive production of clinical-grade material. Heppner *et al.* (41) disagree, and point out that the monkey challenge model is unvalidated. They say that the New World Monkey Challenge model uses an unnatural host, challenged by an unnatural route using an unnatural inoculum. They would prefer to use vaccines produced by Good Manufacturing Practices containing antigens that are recognized by antibodies that have an antiparasitic effect in well-conducted phase 2a studies (studies in a non-endemic area), followed by phase 2b studies in naturally-exposed populations to downselect for future development.

Mouse models, too, have their limitations. No single mouse model displays all the features of human malaria. Nevertheless, the availability of inbred strains, immunological reagents, and transgenic and knockout mice have allowed the identification of protective mechanisms that may operate in human malaria. These studies have been reviewed by Good and Doolan (42), Krzych and Schwenk (43) and Krzych *et al.* (44).

6. PRE-ERYTHROCYTIC VACCINES

To induce sterile immunity, it is necessary to have a pre-erythrocytic vaccine. Pre-erythrocytic vaccines have been reviewed recently (45-47). They may be divided into two groups: sporozoite stage vaccines and liver stage vaccines, though there is an overlap as some antigens are expressed in sporozoites as well as in liver stages.

6.1. Clinical Trials of Pre-erythrocytic vaccines

The subject of clinical trials of malaria vaccines has been reviewed by Greenwood and Alonso (48). The proper sequence of clinical trials, such as whether to test a vaccine in an endemic area before demonstrating efficacy in an experimental challenge, is controversial, and the consensus is evolving (Thomas Richie, Naval Medical Research Center, personal communication, 2006). The sequence may vary depending on the type of vaccine being tested (such as pre-erythrocytic versus blood-stage), the target population, and the outcome of individual phases (48). At the Walter Reed Army Institute of Research, clinical trials are generally done in three stages: a phase 1/2a trial in a non-endemic population and phase 1b and 2b trials in populations in areas where the disease is endemic. This nomenclature will be used throughout the present article for consistency, though it is not used by all workers (48).

Phase 1a and 1b trials are done to determine the safety of a vaccine formulation. The method that our division at WRAIR (Walter Reed Army Institute of Research) uses to challenge volunteers in a phase 2a trial of a pre-erythrocytic vaccine is described in Stoute *et al.* 1997 (49). Volunteers who have never had malaria are immunized and several weeks later exposed to the bites of five mosquitoes that have been infected with chloroquine-sensitive *P. falciparum*. After the mosquitoes have bitten the volunteers, the mosquitoes are dissected and the salivary glands are examined to verify that they contain sporozoites. This challenge technique results in a 97% rate of infection in control volunteers (41). Blood is taken periodically for Giemsa smears to detect parasitemia and for *in vitro* tests of immunity. Volunteers who become infected are immediately treated with chloroquine and other drugs as indicated. Subjects who are asymptomatic and parasite-free for 60 days after challenge are considered to be protected against the disease. An indicator of partial protection is a delay of patency (blood infection). Patency is determined by Giemsa-stained blood smears, but real-time polymerase chain reaction is currently being evaluated for its usefulness in this determination. A two-day or more delay in patency is considered to be a significant difference between control and vaccinated groups.

Clinical trials are also designed to attempt to find correlates of protection, based in part on current thinking as to the mechanisms of immune protection, though microarray technology may allow researchers to find correlates that they did not anticipate and can incorporate into future clinical trials. Rogers and Hoffman (50) point out that different inbred mouse strains show somewhat different mechanisms of protection in response to immunization with irradiated sporozoites, and infer from this that it is likely that individual humans also will use different mechanisms of protection, making it unlikely that a single *in vitro* assay will be predictive of protection in response to all imaginable pre-erythrocytic stage vaccines. Currently, phase 2a trials of pre-erythrocytic vaccines at WRAIR include an ELISA (enzyme-linked immunosorbent assay) for antibodies to epitopes on the challenge antigen, and ELISPOT (enzyme-linked immunospot assay) to measure interferon (IFN) gamma. There are several reasons for including an assay for interferon gamma. Interferon gamma inhibits intrahepatocytic parasite growth *in vitro* (51). Acquired immunity in mice to malaria sporozoites is abolished by anti-IFN-gamma (17) and IFN-gamma receptor knockout mice had a high mortality when challenged with *P. chabaudi chabaudi*, in contrast to wild type mice, which all survived (52). The role of gamma interferon in humans is not certain, but is inferred from its correlation with protection. For example, *in vitro* interferon-gamma responses to merozoite and liver stage antigens are associated with resistance to reinfection to *P. falciparum* in young African children (53). For the LSA-1 (liver-stage antigen-1) trial described below, ELISPOT assays were also used to measure IL-2 responses, because IL-2 is important in the maintenance of memory T cells,

and IL-10, because IL-10 is important in immunoregulation (U. Krzych, WRAIR, personal communication). Because there is a need to identify the correlates of protection, a vaccine trial may include other tests (see, for example, the planned AMA-1 (Apical Membrane Antigen-1) clinical trial described in the section on blood-stage vaccines below). Hermsen and coworkers (54) conducted a phase 1a clinical trial with glutamate-rich protein (GLURP), a protein expressed in both the pre-erythrocytic and erythrocytic stages. The assays that these workers used included lymphocyte proliferation, immunofluorescence on asexual parasites, ELISA, the antibody-dependent cellular inhibition assay (see below), and *in vitro* cytokine production by peripheral blood mononuclear cells.

Once phase 2a studies are completed, vaccines that are being developed for people in endemic areas are tested in phase 1b and 2b studies in endemic areas. A phase 2b clinical trial of pre-erythrocytic vaccines may include both passive and active surveillance (55). The mixture of passive and active surveillance used in a particular trial represents the tradeoff between the high sensitivity required for statistical power and the high specificity required to determine efficacy (56). Phase 2b studies can give very different results from those of phase 2a studies (see the section on TRAP, below) for a variety of reasons, including the fact that in an endemic area, a vaccine must show an enhanced level of protection over pre-existing immunity. Another difficulty in conducting clinical trials in endemic areas is that *in vitro* assays are more difficult to interpret because of preexisting immunity and, for growth-inhibition assays, the possible presence of antimalarial medications in the blood.

6.2. Sporozoite-stage vaccines

An important finding by Nussenzweig and coworkers (57) is that immunization of mice with irradiated *P berghei* sporozoites will elicit sterile immunity. Protection by irradiated sporozoites is also seen in humans, and irradiated sporozoites elicit immunity in human volunteers that lasts at least 10 months and is not limited to a single strain (58). Killed sporozoites do not elicit this response, so it appears that persistence of the parasites within hepatocytes, but not nuclear division, which is prevented by irradiation, is necessary to elicit protective immunity. Vaccination with irradiated sporozoites requires using live mosquitoes to bite humans and release the sporozoites, and has long been considered to be impractical (59). However, a biotechnology company, Sanaria (Rockville, MD) has been established to develop irradiated sporozoite vaccines (60). The success obtained with irradiated sporozoites in the studies described in reference 58 has inspired a variety of vaccine strategies. The first sporozoite surface protein to be identified is circumsporozoite protein (CSP). Because the immune response to sporozoites is largely directed against CSP, many vaccine candidates have incorporated this protein or epitopes from it. Since it appears from measured *in vivo* growth rates (61) that the injection of a single sporozoite can cause malaria, it is often assumed that a vaccine directed exclusively at sporozoites would have to be 100% effective to prevent the disease (62). However, assuming that the number of sporozoites injected follows a Poisson distribution, and that a mean of 5 sporozoites is injected per mosquito bite, a vaccine that is 80% effective (i.e., killed 80% of injected sporozoites) would protect 40% of vaccinees from disease (63). Even if the immunity generated by a vaccine containing sporozoite antigens does not eliminate all of the sporozoites, it may have a second chance at protection, since some sporozoite antigens are present in infected hepatocytes (reviewed in 64). It is believed that T cell responses to circumsporozoite protein are important in the protection induced by sporozoite vaccines. Cytotoxic CD4+ T cells from a sporozoite-immunized volunteer recognize *P. falciparum* CSPprotein (65). A CD4+ cytolytic T cell clone from a mouse that had been immunized with irradiated sporozoites protected mice against *P. berghei* malaria (66).

There are other strategies to mimic the success of vaccination with irradiated sporozoites while avoiding the necessity of growing and purifying large numbers of sporozoites for use in vaccines. For example, identification of the antigens expressed by irradiated sporozoites could allow the development of a vaccine that packages these antigens or the critical epitopes of these antigens (67). Another approach is supported by The Grand Challenges in Global Health Initiative, which has awarded grants to 43 innovative research projects involving scientists in 33 countries. (The initiative's partners are the Bill & Melinda Gates Foundation, the Canadian Institutes of Health Research, the Foundation for the National Institutes of Health, and the Wellcome Trust). In this project (68), by Stefan Kappe's group at Seattle Biomedical Research Institute, a gene in *P. berghei* that is essential for early liver stage development has been inactivated. Immunization of mice with these sporozoites gives complete protection of mice against sporozoite challenge. This vaccine has an advantage over irradiated sporozoites in that the genetic lesion is precise and defined, in contrast to that produced by radiation, and the resulting vaccine is presumably safer and more reliably attenuated (69). Clinical trials of a vaccine containing genetically-modified sporozoites are planned in a collaboration between Kappe's group and WRAIR.

6.2.1. RTS,S

The most successful malaria vaccine candidate that has been tested so far is RTS,S, which is based on the circumsporozoite protein. The RTS,S vaccine has been reviewed recently by Heppner *et al.* (70). The vaccine is a recombinant fusion protein containing the carboxyl-terminal half, amino acids 207-395, of the circumsporozoite protein of *P. falciparum* (strain NF54, clone 3D7), the C-terminal half of CSP comprising 19 copies of the central tetrapeptide repeat sequence NANP, and several T-cell epitopes, and lacks the hydrophobic anchor sequence. The abbreviation "RTS" indicates the presence of the CSP repeat region (R) and T-cell epitopes (T), and the "S" indicates that the RT portion has been fused to the amino terminus of the entire hepatitis B surface antigen (S), a polypeptide of 226 amino acids. The protein is coexpressed in *Saccharomyces cerevisiae* with non-fused hepatitis B surface antigen (HbSAg), and the two

polypeptides spontaneously assemble to form composite particles that are referred to as RTS,S. RTS,S formulated with or without the immunostimulant 3-deacylated monophosphoryl lipid A induced antibodies in mice and monkeys that effectively prevented invasion of cultured human hepatoma cells by *P. falciparum* sporozoites (unpublished data cited in Gordon *et al.* (71). In a Phase 2a study of RTS,S (49), 6 of 6 control volunteers but only 1 of 7 volunteers vaccinated with RTS,S plus in the adjuvant SBAS2 (an oil-in-water emulsion plus monophosphoryl lipid A and QS21, a saponin derivative) became infected.

Alonso and coworkers (55) studied the vaccine RTS,S in the same adjuvant, SBAS2, which had been renamed AS02A, in a phase 2b study of 2022 1-4-year old children in Mozambique. There were two cohorts. In cohort 1, the primary endpoint was time to first clinical episode of malaria and asexual parasitemia over a six-month observation period that began 14 days after the third vaccination. At the end of the observation period, the prevalence of *P. falciparum* infection was 37% lower in the RTS,S/AS02A group compared with the control group. In the RTS,S/AS02A group, 11 of 745 children had at least one episode of severe malaria compared with 26 of 745 children in the control group. Vaccine efficacy for severe malaria was defined according to WHO's guide to clinical practice (72). In a follow-up study (73), in which the children were examined for an additional 12 months, RTS,S/AS02A reduced the risk of clinical malaria by 35% over the entire 18-month observation period with no evidence of waning efficacy.

In vitro assays to determine the correlates of protection may help to identify the nature of the protective immune response elicited by this vaccine. The RTS,S vaccine induces antibodies in volunteers that display antigen-specific opsonizing activity with the THP-1 human monocyte line (74). In addition, protection in RTS,S-immunized volunteers is correlated with the ability of their T cells to make sustained interferon-gamma responses to CSP protein peptides *in vitro* in an ELISPOT assay (75).

Because of the encouraging but partial protection conferred by the RTS,S vaccine, there have been many attempts to improve on it. For example Walsh and coworkers (76) used two DNA plasmid immunizations followed by RTS,S/ AS02A in rhesus monkeys and obtained a better humoral response and a long-lasting CD4+ T cell response. Stewart and coworkers (77, 78) found that a new liposome-based adjuvant, AS01B, which contains the same components as AS02A (which is an oil-in water emulsion), gives better interferon gamma ELISPOT responses and larger and longer-lasting delayed-type hypersensitivity responses than AS02A.

A systematic review of clinical trials of pre-erythrocytic vaccines for malaria (79), including 4 safety/efficacy trials and 2 safety trials of RTS,S, concluded "Progression of this vaccine towards licensing is justified while efforts to increase its efficacy continue." The RTS,S vaccine is currently being tested in Ghana, Kenya, Sierra Leone, Gabon, Tanzania, and Senegal (80). Meanwhile, promising new formulations of the vaccine are being tested in monkeys (Ann Stewart, WRAIR, personal communication, 2006).

6.2.2. TRAP

The second parasite-encoded molecule to be identified on the surface of salivary gland sporozoites was sporozoite surface protein 2 (81). A sporozoite surface molecule with a different name, TRAP (thrombospondin-related adhesion protein or thrombospondin-related anonymous protein), turned out to be the same molecule as sporozoite surface protein 2 (82). Sultan and coworkers (83) by disrupting the *P. berghei* TRAP gene, found that TRAP is necessary for the gliding motility of sporozoites *in vitro* and the infection by sporozoites of the mosquito salivary gland and the rat liver. Khusmith and coworkers (84) obtained complete protection of BALB/c mice against a challenge with *Plasmodium yoelii* sporozoites by immunizing the mice with P815 cells that had been transfected with the *P. yoelii* SSP2 and CSP genes. Schneider and coworkers (85) used plasmid DNA encoding the TRAP antigen, followed by a single boost with modified vaccinia virus Ankara encoding the same antigen, to immunize mice, and obtained complete protection against *P. berghei* sporozoite challenge. Müller and coworkers (86) found that mouse antisera raised to TRAP constructs inhibited sporozoite invasion of human hepatoma HepG2 cells.

Walsh and coworkers (87) found that a combination of RTS,S and TRAP was safe and immunogenic in infant rhesus monkeys. McConkey and coworkers (88) tested a vaccine construct called "ME-TRAP" in a phase 1/2a trial with malaria-naïve adult volunteers. ME (multiple epitope) is a string of 14 CD8+ T cell epitopes, one CD4+ T cell epitope, and two B cell epitopes from six pre-erythrocytic *P. falciparum* antigens. It also contains two non-malarial CD4+ T cell epitopes. They used a heterologous prime-boost vaccination regime of DNA either intramuscularly or intradermally, followed by intradermal recombinant modified vaccinia virus Ankara (MVA). This vaccine regimen induced high frequencies of interferon-gamma-secreting, antigen-specific T cell responses to TRAP, and partial protection manifested as delayed parasitemia after sporozoite challenge with a different strain of *P. falciparum*.

Dunachie and coworkers (89) tested TRAP and CSP in a phase 2a trial of human volunteers using heterologous prime-boost regimens. In the first group, two DNA-ME TRAP vaccinations were followed by one MVA-ME TRAP vaccination, and in the other group, two DNA CSP vaccinations were followed by MVA encoding CSP. Volunteers were challenged by being bitten by *Anopheles stephensi* mosquitoes infected with *P. falciparum* parasites. Six of six control subjects, seven of eight TRAP subjects, and eight of eight CSP subjects were diagnosed with malaria (parasitemia detectable microscopically or by real-time PCR). The DNA-ME TRAP group showed a significant delay to parasitemia compared with the control group, but the CSP group did not.

Moorthy and coworkers (90) followed up this study with a phase 2b trial of ME-TRAP with 372 men in

The Gambia. Two vaccine vectors encoding ME-TRAP, plasmid DNA and modified vaccinia virus Ankara (MVA) were used sequentially in a prime-boost immunization regime. The vaccine regimen was safe and well-tolerated, and was strongly immunogenic as measured by a gamma-interferon ELISPOT assay. However, it was ineffective in reducing the natural infection rate in semi-immune African adults. This conclusion was based on an endpoint of time to first infection with asexual *P. falciparum,* defined as the number of days from the start of the surveillance period to the first positive slide. The results were surprising after having seen partial protection in the earlier study. Moorthy *et al.* note that use of surrogate endpoints may lead to negative results and may incorrectly lead to the cessation of development of a candidate vaccine. They believe that protection against disease may still be possible with this vaccine.

Kester and coworkers (WRAIR, personal communication, 2006) tested a combination of RTS,S and TRAP and TRAP alone in a clinical trial. These candidate vaccines had been tested previously in rhesus monkeys (87). Both RTS,S/TRAP and TRAP were safe and immunogenic. There was no protection with either group, suggesting that TRAP itself did not provide any pre-erythrocytic protection and that the addition of TRAP to RTS,S negated the protective efficacy induced by RTS,S alone.

6.3. Liver-stage vaccines

Liver-stage vaccines have been reviewed by Hollingdale *et al.* (91) and Hollingdale and Krzych (17), and the role of T cells in immunity to malaria liver stages has been reviewed by Morrot and Zavala (92), and Krzych and Schwenk (43).

The assays used to evaluate sporozoite and liver-stage vaccines include ELISPOT and intracellular staining for cytokines. These assays are done because it is believed that CD8+ T cells play a role in the protection of humans against malaria. This belief is based in part on mouse studies and in part on indirect evidence from some human studies. Not all human studies support the hypothesis that CD8+ cells are protective against malaria (see below). Cell mediated immune responses, primarily CD4+ and CD8+ T cell responses, are implicated in protection against the liver stage of murine malaria (reviewed in 44). CD8+ T cells that recognize malaria antigens on major histocompatibility complex class I molecules on hepatocytes may play a role in murine immunity (93, 94). CD8+ T cells may act by killing parasite-infected hepatocytes, a phenomenon that has been demonstrated *in vitro* (95), or by the production of interferon-gamma (see below). The relative importance of the cytotoxic and cytokine-producing activities of CD8+ T cells in immunity to malaria is not known. Immunity can be obtained in perforin-knockout mice immunized with irradiated sporozoites (96), but anti-sporozoite antibodies or CD4+ T cells may have played a role in these experiments. Alpha-beta T cells have a major role in protection in sporozoite-immunized mice, and gamma-delta T cells contribute to the inhibition of the early stages of development of hepatic parasites (reviewed in 64). With mice, it is possible to do adoptive transfer studies and to unambiguously show that CD8+ T cells are involved in protection against malaria, (92, 94, 97) while in humans, the role of CD8+ cells in protection is inferred from the correlation of high frequencies of antigen-specific CD8+ cells or certain HLA types with protection (75, 88, 92, 98), and is therefore suggestive but not certain. If CD8+ T cells are indeed involved in protection, a likely mechanism, not necessarily the only mechanism, is via their production of interferon gamma and the resulting production of nitric oxide by macrophages. This is suggested by animal data (42) and by several human studies. For example, Anstey and coworkers (99) found an inverse relationship between nitric oxide synthase activity and malaria severity in children in Tanzania. There is also evidence for T cell involvement, nitric oxide synthase activity, and IFN-gamma production in human protection against blood stage infection (ref 100; see section 7.1). On the other hand, Wang and coworkers (ref. 101, see section 12.2) obtained CD4+ and CD8+ cell responses in volunteers immunized with a DNA vaccine encoding five pre-erythrocytic antigens, but none of the volunteers was protected, and as noted in section 6.2.2 on TRAP above (90), a phase 2b trial showed a good ELISPOT response but no protection against infection.

6.3.1. LSA-1

Liver stage antigen 1 (LSA-1) is a 230-kDa protein found within the parasitophorous vacuole surrounding *P. falciparum* exoerythrocytic parasites (102, 103). The exact function of the protein is unknown (104). Studies of its role in immunity to malaria have been limited to humans because no homologue has been found in murine or non-human primate malaria parasites. In several studies of individuals immunized with irradiated sporozoites or exposed naturally to malaria, T cell responses to LSA-1 correlated with protection (reviewed in 105). The preparation of LSA-1 for clinical studies by good manufacturing practices is described by Hillier *et al.* (104). This construct, which was expressed in *Escherichia coli* with a gene that was developed using codon harmonization (see section 11, below), has two repeats of a 17 amino acid sequence (which is present in the native antigen in about 87 repeats) as well as the highly conserved N-terminal and C-terminal regions, which are known to contain both B- and T-cell stimulating epitopes. A phase 1/2a clinical trial in which LSA-1 was injected the adjuvants AS01B in one group of volunteers and with AS02A in the other group has recently been carried out in our division at the Walter Reed Army Institute of Research (J. Cummings, WRAIR, personal communication, 2006). The adjuvants are from GlaxoSmithKline (GSK) Biologicals (Rixensart, Belgium). There were no serious adverse events (serious reactions) with either mixture, and many of the volunteers made antibodies to LSA-1 that were detectable by ELISA. The vaccination did not show any efficacy as measured as protection against infection or a delay in the onset of infection.

6.3.2. LSA-3

Liver stage antigen 3 (LSA-3) was identified by Daubersies and coworkers (39) by screening 120 phage lambda clones expressing *P. falciparum* pre-erythrocytic

proteins with sera obtained from humans and chimpanzees immunized with irradiated sporozoites. LSA-3 has a predicted molecular weight of 200 kDa, is highly conserved, and is expressed in both mosquito and liver-stage parasites. LSA-3 is thought to have a role in liver schizogony and merozoite release (106). Daubersies and coworkers (39) immunized chimpanzees (*Pan troglodytes*) with LSA-3 and LSA-3 peptides in various adjuvants. Among four chimpanzees receiving LSA-3 emulsified in the adjuvant SBAS2, two chimpanzees showed full, sterile protection (no parasites in the blood) 5-10 days after intravenous challenge with *P. falciparum* sporozoites, one showed a one-day delay in patency, and one showed no protection.

7. BLOOD-STAGE VACCINES

Blood stage vaccines have been reviewed recently by Good *et al.* (107). The blood stage of the *Plasmodium* life cycle is responsible for the symptoms of malaria, including fever, chills, lethargy, and organ failure and death in some individuals. A blood-stage vaccine could be very effective in reducing morbidity and mortality for people in endemic areas even if it does not eliminate all parasites because many people in endemic areas have partial immunity and therefore have few or no symptoms of disease despite the fact that many of them have a low level of parasitemia.

7.1. Clinical trials of blood-stage vaccines

A human test using *Plasmodium*-infected red blood cells to measure the effect of blood-stage vaccines on *Plasmodium* growth rates has been described by Cheng *et al.* (61). The blood used in this type of challenge must be carefully tested for the presence of other pathogens. At WRAIR, phase 2a clinical trials of blood-stage vaccines employ the same mosquito challenge model that is used for trials of pre-erythrocytic vaccines. The goal of giving blood-stage vaccines is to reduce the parasite burden, rather than induce sterile immunity. However, in a phase 2a trial, in which volunteers are not parasitemic at the start of the trial, control and vaccinated groups are compared with respect to the onset of patency. As with pre-erythrocytic vaccines, a delay in patency of at least two days is considered to be significant. Because some antigens that are present in the blood stage and are part of candidate blood-stage vaccines, such as AMA-1, are also present in earlier stages of the parasite, this challenge model does not indicate which stage is being targeted by a vaccine.

In Phase 2b trials of blood-stage vaccines, some of the volunteers may be parasitemic and asymptomatic before the trial starts. In that case, they may be given an antimalarial drug to clear the parasites. This treatment makes it possible to measure delay in patency. However, in some cases the use of antimalarial drugs may obscure the protective effect of a vaccine (see the trial of Genton *et al.* in section 7.4). The problems associated with establishing a case definition of malaria for clinical trials are discussed in Section 16 below.

Because red blood cells lack major histocompatibility complex (MHC) antigens, the classic MHC-restricted CD8+ T cell-mediated lysis of infected red cells is unlikely, and blood-stage vaccine strategies have for the most part focused on eliciting antibody production. A variety of assays for anti-malarial antibodies are in use, though without a detailed knowledge of the mechanism of protection, it is impossible at present to choose one assay over the others. The enzyme-linked immunosorbent assay (ELISA) is well-understood, is widely-used for testing antibody responses, and can be precisely standardized. ELISA titers to certain antigens often correlate with protection against malaria (108, 109). However, it detects antibodies with 10-100-fold lower avidity than that of protective antibodies (reviewed in 24). Indirect immunofluorescent (IFA) staining of parasite-infected red blood cells is a way to check if antibodies elicited by a vaccine react with native proteins, and therefore to verify that the protein used in the vaccine is correctly folded. Assays to detect the inhibition of growth of parasites in red blood cells have been developed in several laboratories (110-112). Four growth-inhibition assays have been compared using the same antibodies by Bergmann-Leitner and coworkers (113). These authors conclude that assays must determine both parasitemia and viability to detect the full range of anti-parasite activities in immune sera. Another assay, which depends on the action of antibodies and monocytes, called the antibody-dependent cellular inhibition assay, is also used to study antibody responses (114, 115). It is not clear which of these assays is the best or how useful they will prove to be. Different assays may be appropriate for different vaccines. Potency assay design for malaria vaccines consisting of adjuvanted recombinant proteins is reviewed in ref. 116.

Despite the focus on antibodies in assays for protection against blood stage malaria, Su and Stevenson (117) showed using knockout mice that interferon gamma also important in resistance to blood stage infection. Interferon gamma enhances the production of protective cytokines such as TNF-alpha and inhibits the production of inhibitory cytokines such as interleukin-10, and also changes the isotype profile in ways that may enhance macrophage-mediated phagocytosis (117). There is also evidence from a human trial. Pombo and coworkers (100) immunized human volunteers by inoculating them repeatedly with red cells infected with *P. falciparum* and curing the infection with drugs. The volunteers were protected against subsequent challenge with approximately 30 malaria-infected red blood cells. After immunization, the volunteers had no demonstrable antibodies to *P. falciparum,* but there was an increase in CD4+ and CD8+ T cell proliferation in response to antigen, and an increase in IFN-gamma production and nitric oxide synthase activity.

7.2. AMA-1

Apical membrane antigen-1 (AMA-1) is a merozoite protein that is expressed during the asexual blood stages of *P. falciparum*, AMA-1 is an 83-kDa protein characterized by eight intramolecular disulfide bonds and is located in the apical micronemes of the merozoite (118, 119). Studies using gene substitution suggest that AMA-1 is critical for the invasion of red blood cells by merozoites (120). Mitchell *et al.* (121) found that AMA-1 mediates the

close attachment of invasive merozoites to host red blood cell. AMA-1 in *P. falciparum* undergoes proteolytic processing in the parasite after translocation from the micronemes to the parasite surface (122). The main chain is cut 29 residues upstream from the transmembrane region, this cleaving the ectoplasmic region from the merozoite surface. The mode of action of invasion-inhibitory anti-AMA-1 antibodies appears to be inhibition of the processing of AMA-1 (123). Immunization of mice with AMA-1 protects mice against lethal infection with *P. yoelii yoelii* (124). Stowers *et al.* (38) found that *Aotus vociferans* monkeys vaccinated with *P. falciparum* AMA-1 were protected against blood-stage malaria. In a phase 1a clinical trial of an AMA-1 vaccine, 23 out of 25 (92%) of vaccines made IgG anti-AMA-1 antibodies that were detectable in an ELISA (125). Antigen-specific *in vitro* inhibition of both the FVO and 3D7 strains of *P. falciparum* was seen in IgG purified from sera of the vaccines (125). A phase 1/2a clinical trial of the 3D7 allele of AMA-1 with the adjuvants AS01B/AS02A from GSK is under way in our division (J. Cummings and M. Spring, WRAIR, personal communication, 2006). Assays to be done for correlates of protection include growth inhibition assays (110, 111), a processing-inhibition assay (123) and real-time PCR for parasitemia (M. Spring, personal communication).

There are concerns that allelic variation in AMA-1 (apical membrane antigen-1) and MSP-1 (merozoite surface protein-1), may compromise the effectiveness of vaccines to these proteins (126). Our approach to allelic variation is to use the simplest vaccine that will work, which in the case of AMA-1 means to vaccinate with a single allele and determine in a phase 2b trial whether the protection is allele-specific. The FVO and 3D7 strains of *P. falciparum* express different alleles of MSP-1 and AMA-1. If the protection given by vaccinating with one allele is allele-specific, our group will try vaccinating with the other allele. Only if both alleles give allele-specific protection will we include more than one allele in a vaccine (D. Gray Heppner, WRAIR, personal communication, 2006). The preparation of a GMP-grade, properly-folded antigen is very laborious and time-consuming, and, in addition, optimizing the dose and timing of a vaccine containing more than one antigen is very difficult, so it is not always desirable to delay clinical trials until antigens of all alleles are available.

7.3. MSP-1

The merozoite-surface protein 1 (MSP-1) complex of *Plasmodium falciparum* constitutes a major component at the surface of the erythrocyte-invading form of the parasite (127). It originates from an approximately 190-kDa glycosylphosphatidylinositol (GPI)-anchored precursor, which is proteolytically processed during merozoite maturation, yielding in a first step four major fragments, p83, p30, p38, and p42, which remain noncovalently associated (128). After merozoite release, a secondary processing event occurs in which the membrane-bound 42-kDa component, of this complex is cleaved to a 33-kDa fragment, which is shed, and a C-terminal fragment, p19 (also known as MSP-1_{19}), which remains on the merozoite. Preventing the proteolytic cleavage that generates p19 inhibits invasion of red blood cells *in vitro* (129).

In several studies, a vaccine containing MSP-1 purified from *P. falciparum* partially (130) or completely (131) protected against challenge in primate models (reviewed in 132). Individuals with high levels of antibodies to MSP-1_{19} that inhibit invasion *in vitro* appear to be protected against blood-stage malaria infection in western Kenya (133) but in The Gambia, the only anti-MSP-1_{19} antibodies that were associated with protection (lower parasite density) were those that competed with an invasion-inhibitory monoclonal antibody (134). In a phase 1a clinical trial, Ockenhouse and coworkers (135) vaccinated 15 malaria-naïve adult volunteers with the FMP1 (falciparum malaria protein) antigen, a His-tagged fusion protein from the 42 kDa C-terminal fragment of the 3D7 allele of MSP-1. The vaccine was safe and well-tolerated and no serious adverse events were observed. The vaccine induced high-titer ELISA responses against FMP1 antigen and antibodies in all volunteers that reacted in an immunofluorescence assay with air-dried and methanol-fixed schizonts. The vaccine induced antibodies that killed parasites in an *in vitro* growth inhibition assay Interferon-gamma secretion was observed in ELISPOT assays of blood from immunized volunteers, and proliferative responses to the FMP1 antigen were observed in >80% of volunteers. Stoute and coworkers (136) studied FMP1/AS02A in a phase 1b clinical trial in adults in Kombewa Division, western Kenya. The vaccine was safe and caused an increase in antibody titers (above a high level of pre-existing antibody) to MSP-1_{42} by ELISA. Withers and coworkers (137) conducted a phase 1b clinical trial of the same vaccine in children in the same region of western Kenya. The vaccine was safe and well-tolerated. Anti-FMP1 antibody titers were higher in the vaccinated group than in the control group, and were positively correlated with the dose of vaccine.

7.4. MSP-1, MSP-2, and RESA

The ring-infected erythrocyte surface antigen (RESA) was recognized simultaneously by Perlmann and coworkers (138), and by Coppel and coworkers (139). It is a 155 kDa protein found on the surface of human erythrocytes infected with *P. falciparum*). Antibodies to RESA inhibit merozoite invasion into erythrocytes (140) though the exact role of RESA in the life of the parasite is not known. Candidate RESA vaccines gave varying results in monkey challenge studies. A trial in which *Aotus vociferans* monkeys were immunized with *E. coli*-derived fused polypeptides corresponding to various regions of the RESA molecule showed some protection against high-level parasitemia after intravenous challenge with *P. falciparum* (141). A subsequent challenge of *Aotus nancymai* by the same group (142) showed no protection, as judged by maximum levels of parasitemia. The reason for the different results was not known, but the authors suggested that different species of monkeys, different adjuvants, or different strains of *P. falciparum* might have explained the differences. Another study (143), using *Saimiri sciurus* monkeys and recombinant vaccinia viruses containing the

gene for the entire RESA antigen or peptides of RESA, showed little or no antibody production against RESA and no protection. Despite the variable monkey responses, there were reasons to continue to test the RESA antigen. These include the inhibition studies mentioned above as well as epidemiological studies. For example, RESA is recognized by antibodies from malaria patients and immune individuals (138). The N-terminal region of RESA contains several B- and T-cell epitopes that are recognized by antibodies and cells from many malaria-immune donors in Senegal (144). Also, antibody responses (145) and T cell responses (146) to RESA are associated with protection from clinical malaria in children in Papua New Guinea.

Genton and coworkers (147, 148) tested a three-component blood stage vaccine, MSP-1, MSP-2, and RESA, which is known as "Combination B", with the adjuvant Montanide ISA 720 in a phase 1/2b clinical trial in children aged 5-9 years in Papua New Guinea. This was the first trial of a malaria vaccine containing only blood-stage components in a previously-exposed population. No serious adverse events occurred. The vaccine induced significant IgG antibody responses, as measured by ELISA, to all three antigens. Cytokine responses were measured by testing the supernatant of peripheral blood mononuclear cells stimulated *in vitro* with the various antigens. An interferon-gamma response was only seen in response to MSP-1. To test the efficacy of the vaccine, parasite density was measured because it was expected to be statistically more powerful than measuring morbidity. Half the children were pretreated with sulfadoxine/pyrimethamine (SP) before immunization and the other half were not. The vaccination resulted in a 62% reduction in parasitemia in children not pretreated with SP, but no significant difference in SP-pretreated children. Substantially fewer high parasitemias (parasite densities >500/microliter) occurred in the no-SP group. There was no significant effect on the incidence of clinical malaria episodes. In a review of clinical trials of combination B vaccines, including this one, Graves and Gelband (149) point out that pretreatment with sulfadoxine/pyrimethamine clearly reduces infection, allowing the incidence of new infections to be more easily determined, but this strategy also reduces the power to detect changes in prevalence.

There are two allelic families of MSP-2, FC27 and 3D7. Only the 3D7 form of MSP-2 was included in the vaccine. When PCR was used to analyze the blood samples of vaccinees, the prevalence of the 3D7 form of MSP-2 was significantly reduced, while the prevalence of FC27-type parasites was not. This result implies that vaccine formulations must include both allelic forms of MSP-2. The literature review (149) of Combination B clinical trials concluded that this vaccine "shows promise."

7.5. MSP-3

Merozoite Surface Protein-3 (MSP-3) is a protein of 386 amino acids that was identified by screening protected individuals for antibodies that cooperated with monocytes in killing parasites *in vitro* (150). In children in Burkina Faso, a positive association was found between IgG antibody levels against the MSP-3 long synthetic peptide 154-249 and protection from clinical malaria episodes (151). A vaccine based on MSP-3 was tested in *Aotus nancymai* monkeys that were challenged intravenously with *P. falciparum*-infected erythrocytes (152). Protection was defined as the ability to keep their parasitemia below 5%. Five of seven control monkeys had a fulminant infection and the remaining two required treatment for anemia. Five of seven monkeys vaccinated with MSP-3 vaccine were protected, one required treatment for parasitemia, and one died of heart failure. In a phase 1a clinical trial, an MSP-3 vaccine formulated with Montanide ISA720 or alum was immunogenic, with a majority of volunteers producing anti-MSP3 antibodies that were active in the antibody-dependent cellular inhibition assay (150). A majority of volunteers also showed a T cell proliferative response and interferon gamma production (150). Antibody to the native protein was seen in the sera of 60% of the volunteers, and this antibody activity was related to the ability of the sera to reduce or abrogate parasitemia in humanized SCID mice infected with *P. falciparum* (153). With alum, the antibody remained in samples taken up to 12 months postimmunization. However, the vaccine was unacceptably reactogenic when combined with the adjuvant Montanide ISA 720 (150).

8. TRANSMISSION-BLOCKING VACCINES

Transmission-blocking vaccines kill the gametocytes that are formed in the blood of the human host or prevent fertilization or the development of the parasite within the mosquito (reviewed in 154 and 155). Since the asexual blood-stage cycle is not targeted, these vaccines would not be expected to protect the individual who is vaccinated but instead would protect people who live in the immediate area. The indication for transmission-blocking vaccines will likely be solely for people living in malaria-endemic or malaria-epidemic regions (154). The feasibility of transmission-blocking vaccines is suggested by the fact that adults in endemic areas produce antibodies that reduce the efficiency of *Plasmodium vivax* transmission to the mosquito, as determined by the membrane-feeding test (156).

For malaria transmission, asexual parasites in the human host must differentiate into mature gametocytes that can be taken up by a mosquito when it takes a blood meal. After a few minutes in the mosquito midgut, the gametocytes are stimulated to emerge from the red blood cells and the males exflagellate, producing eight haploid motile microgametes that fertilize female gametes (macrogametes) to produce zygotes. During the next 24h, the non-motile zygotes differentiate into motile ookinetes that can penetrate through the midgut epithelium and develop into oocysts in the extracellular space between the midgut epithelium and the overlying basal lamina. Ten to 24 days after infection, thousands of sporozoites are produced within each oocyst and when the oocyst ruptures they are released into the hemocoel (157). As they circulate past the salivary glands the sporozoites bind and invade so that when the mosquito takes its next blood meal it releases saliva containing infectious sporozoites into the

vertebrate host. Several candidate antigens for transmission-blocking vaccines have been identified. Pfs48/45, a surface protein which is expressed by male and female gametocytes of *P. falciparum*, has a role in fertilization, as shown by gene disruption studies (158). Gene disruption studies suggest that Pfs230, which is also found on the surface of gametes, is involved in the binding of microgametes to red blood cells to form clusters called exflagellation centers (159). Individual motile microgametes are released from these centers to find and fertilize macrogametes to produce zygotes. Ookinete surface proteins Pfs25 and Pfs28 are also potential antigens for transmission-blocking vaccines. In the midgut of mosquitoes, the formation of ookinetes lacking both proteins (double knockouts) is significantly inhibited due to decreased protection from lethal factors, including protease attack, and double knockout ookinetes have a much reduced capacity to traverse the midgut epithelium and to transform into the oocyst stage (160).

Pfs230 and Pfs48/45 are expressed on the plasma membrane of gametocytes as they develop inside the human host, while the expression of Pfs25 and Pfs28 is limited to the mosquito stage of the parasite's life cycle (155). Their expression begins after the parasite emerges from the red blood cell as a gamete and continues through ookinete development (155). These facts imply certain advantages and disadvantages to the choice of antigens for transmission-blocking vaccines. On the one hand, Pfs230 and Pfs48/45 would be subjected to selective pressure due to the immune response of the human host, while Pfs25 and Pfs28 presumably would not, so the latter two antigens might be chosen because they would be expected to show less variability from strain to strain. On the other hand, it might be desirable to choose Pfs230 or Pfs48/45 because they are expressed in the human host and therefore the immune response will be boosted by natural exposure to malaria parasites. Two other potential antigens associated with the late midgut stage have been identified: chitinase and mosquito-produced protease (161).

Several animal studies have provided support for the concept of transmission-blocking vaccines. Arakawa (162) immunized mice with the ookinete surface protein Pfs25. Feeding *Anopheles dirus* mosquitoes with a mixture of mouse immune serum and gametocyte-containing blood derived from patients infected with *P. falciparum* resulted in complete interference with oocyst development, as determined by a microscopic count of oocysts in the midguts. Stowers (163) vaccinated mice with one of four epidermal growth factor-like domains of the *P. falciparum* sexual-stage antigen Pfs25 and boosted them with the full-length protein. Sera from these mice completely blocked the development of oocysts in mosquito midguts in membrane-feeding assays. Duffy and Kaslow showed that antiserum raised against yeast-secreted recombinant Pfs28 elicits transmission-blocking activity in laboratory animals (unpublished data of P.E. Duffy and D.C. Kaslow cited in 161). A phase I clinical trial in which a viral vector prime (NYVAC-Pf7) was boosted with TBV25H/alum (TBVS25H is *P. falciparum* Pfs25 with a histidine tag) showed transmission-blocking activity in a membrane-feeding assay (154). The midguts of mosquitoes that had fed on serum taken from the immunized individuals after a booster vaccination had lower frequencies of oocysts than mosquitoes fed on serum taken before the booster.

Sample sizes for phase 2b clinical trials of transmission-blocking vaccines have been discussed in a review by Kaslow (161). The calculation is unconventional because the people who are vaccinated are not the ones who are expected to be protected. Therefore, the rate of infection, the rate of gametocyte production, and the proportion of people with gametocytemia who transmit the parasite to mosquitoes all influence the required sample size. In some areas of the world, the required sample size would be in the low hundreds or less and a phase 2b trial is therefore considered to be feasible.

9. VACCINES AGAINST MEDIATORS OF THE DISEASE PROCESS

The natural adaptive immune response to malaria infection can provide partial, but incomplete, protection against pathogen replication, but the immunological reactions can also contribute to disease and fatalities, and this fact has implications for vaccine design (reviewed in 164). A possible advantage of eliciting what is called anti-toxic immunity is the apparent lack of antigenic variation seen in experiments in which mice were immunized with toxic molecules and protected against challenge with *P. yoelii*, which suggests that a whole series of species and variant-specific antigens would not be needed in a vaccine (reviewed in 165). Antitoxic immunity to malaria has been reviewed by Schofield (166). The possibility that anti-disease vaccines will cause increased virulence is discussed in section 15 and the references cited therein.

One potential target of antitoxic immunity is *P. falciparum* glycosylphosphatidylinositols, which can induce the pathophysiology ascribed to putative malaria toxins (reviewed in 167). Schofield (168) immunized C57BL6/J mice with deacylated synthetic *P. falciparum* GPI glycan conjugated to keyhole limpet hemocyanin and reduced the early mortality caused by a challenge with *P. berghei* (ANKA strain) from 91-100% to 25-52%. Antibodies from mice immunized with keyhole limpet hemocyanin-glycan inhibited the production of tumor necrosis factor by macrophages stimulated with *Plasmodium* schizont extract. Elevated levels of tumor necrosis factor (TNF) are associated with severity of disease and death in patients with cerebral malaria (see review in 165). TNF is also associated with protection against the parasite (169). Therefore, antibodies to TNF or inhibitors of TNF may have undesirable effects.

10. VACCINES FOR MALARIA DURING PREGNANCY

There is an increase in the severity of malaria during pregnancy, resulting in increases in maternal and infant mortality (reviewed in 25 and 170). The increased vulnerability of both mother and developing child to malaria results from the specific adherence and

accumulation of parasite-infected erythrocytes within the placenta and a potentially important localized immune response (171, 172). The primary molecule responsible for the adherence of infected red cells to the placenta is believed to be *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is also responsible for the adherence of red cells to the deep vascular beds of many tissues. It is encoded by the multicopy *var* gene family. Individual parasites express a single *var* gene, but populations of parasites can switch *var* gene expression and thereby evade the immune response (173). Salanti and coworkers (174) found that a single *var* gene, var2csa, is selectively upregulated in pregnancy-associated malaria, and Duffy and coworkers(175) found that the gene product, VAR2CSA, is the principal ligand for chondroitin sulfate A in the placenta. A vaccine based on this molecule may be effective (176). Baruch and coworkers (177) vaccinated *Aotus* monkeys with a functional domain of one variant of PfEMP1 and observed protection against the homologous strain of *P. falciparum*. There was no protection, but there was a boosting of the immune response, when the monkeys were challenged with a different *P. falciparum* strain, suggesting that there are cross-reactive regions in this polymorphic molecule that may be useful in vaccines.

Snow and coworkers (178) found a decreased risk of malaria in areas of perennial transmission up to the third month of life and in areas of seasonal transmission up to the sixth month of life. They suggested several possible explanations for this phenomenon, including protection from maternally-derived IgG. However, Riley and coworkers (179) examined IgG antibody levels at birth to five malaria antigens and found that, rather than being associated with resistance to infection, the antibodies were positively associated with infection. The question of protection of infants from malaria by maternal antibodies has been reviewed by Riley *et al.* (180). These authors conclude that "[T]he evidence to date indicates that maternally derived antibodies have little if any effect on development of patent blood stage malaria in infants. However, the question of whether maternally derived antibodies can protect young babies from high-density infection and clinical disease has not really been resolved." Another possible mechanism for the protection of neonates was described by King *et al.* (181), who showed that cord blood from some infants born in areas of stable malaria transmission had T and B cells that were sensitized to MSP-1_{19} or epitopes of MSP-1_{19} and also had IgM anti-MSP-1_{19} antibodies. This result suggests that the protection of neonates results at least in part from prenatal sensitization to malaria antigens.

11. PROBLEMS IN MAKING RECOMBINANT PROTEIN VACCINES

The *Plasmodium* genome is highly A+T-rich (58) and thus the concomitant codon usage bias that exists can lead to difficulties in heterologous expression. One method to optimize expression is codon optimization, in which codons having the highest usage frequencies for the expression host are substituted throughout the gene sequence (182). One potential problem with this approach is that the resulting proteins may not be folded properly. To solve this problem, a new algorithm was developed termed codon harmonization (E. Angov, C. J. Hillier, R.L. Kincaid and J.A. Lyon, WRAIR, manuscript in preparation). For this approach, the algorithm makes use of codon frequency tables for the expression host (e.g., *E. coli*) and the natural host of the gene (e.g. *P. falciparum*). The algorithm substitutes natural host codons with synonymous expression host codons having similar or identical usage frequencies. For example, codons that are used frequently in *P. falciparum* are substituted by synonymous codons that are used frequently in *E. coli*. Since the frequency of codon usage correlates with the rate of translation, recoding gene sequences by codon harmonization ensures that translational pauses occur at the same places that they would occur in the native host, and thus allows for folding to proceed properly. This approach has been applied to a recombinant LSA-1 (3D7 allele) malaria vaccine candidate as well as to three alleles of MSP-$1_{42,}$ the 42 kDa C-terminal portion of MSP-1. In all cases where the algorithm has been applied, the outcome has been high levels of soluble protein, and for MSP-$1_{42,}$ the purified recombinant proteins were reactive with conformation-dependent antibodies made against *Plasmodium* parasites, suggesting the acquisition of some correct structure.

12. DNA VACCINES

12.1. Rationale for DNA vaccines

DNA vaccines for malaria have been reviewed by Tuteja (183). DNA vaccines are attractive because of their simplicity of design, modification, and large-scale production, ease of combining several antigens, stability, lack of requirement for a cold chain, and their ability to induce CD8+ T cell responses as well as antibody responses (Doolan and Hoffman (184). There is no doubt that CD8+ T cells play a role in protecting mice from malaria, but the evidence for a role for CD8+ T cells in the protection of humans from malaria is not definitive, and the results of different studies are seemingly contradictory (see section 6.3 above). Nevertheless, some vaccines, such as ME-TRAP, are deliberately designed to contain CD8+ T cell epitopes.

12.2. Heterologous prime-boost strategy

Vaccination with DNA encoding protective antigens has elicited strong cell-mediated responses in mice (185), but humans do not respond as strongly as mice to DNA vaccine alone (186, 187). A variety of strategies have been tested to overcome this problem. For example, DNA vaccines can induce a T cell response that can be boosted by attenuated viral vectors encoding the same antigen, or by the protein antigen in adjuvant. In some experiments, one virus encoding a test antigen is used for the priming immunization and another virus encoding the same antigen is used for the booster. The viral vectors have to be chosen with care because anti-vector immunity can reduce the efficacy of a vaccine (188). Prime-boost immunization strategies have been reviewed in refs 189-191. Several prime-boost immunization studies were described above in the section on TRAP. One of them was the study of Webster and coworkers (192) in which a

regimen of two viruses, FP9 and MVA, gave good responses to TRAP. In contrast, Walther and coworkers (193) used the same promising regimen of FP9 followed by MVA, this time encoding full-length circumsporozoite protein, and obtained no protection and modest T cell-responses. In another approach, Walsh and coworkers (76) used a CSP-encoding DNA plasmid followed by RTS,S/AS02A in monkeys. This regimen gave long-lasting CD4+ T cell responses (as measured by delayed-type hypersensitivity) and antibody responses to the C-terminus of the circumsporozoite protein.

DNA vaccines can be used to deliver a group of antigens in a single immunization. For example, Prieur and coworkers (194) constructed a candidate vaccine expressing six preerythrocytic antigens, linked together to produce a 3,240-amino acid-long polyprotein, which was expressed by a plasmid DNA vaccine vector and by modified vaccinia virus Ankara and by the FP9 strain of fowlpox. Interferon gamma-secreting T cells specific for each of the six antigens were induced after vaccination of mice. However, the DNA encoding the polyprotein was not capable of priming the mice; it could only boost a response to a single antigen in mice that had been primed with DNA encoding only that antigen. Prime-boost regimens in which MVA viruses encoding the polyprotein were boosted with FP9 viruses encoding the polyprotein, and vice versa, were able to prime CD8+ T cell responses (as measured by tetramer binding) to LSA-1.

A phase 1/2a clinical trial of a group of five plasmids encoding five pre-erythrocytic stage antigens was described by Wang *et al.* (101). The plasmids encoded MHC class I-restricted epitopes from the antigens PfCSP (*P. falciparum* circumsporozoite protein), PfSSP2, PfEXP-1 (exported protein-1), PfLSA-1, and PfLSA-3. Along with these plasmids, another plasmid encoding human granulocyte-macrophage colony-stimulating factor was injected. After DNA immunization, antigen-specific gamma interferon responses were detected by the ELISPOT technique in 15/31 volunteers to multiple class I and/or class II-restricted T cell epitopes from all five antigens. Volunteers were challenged with sporozoites via the bites of infected *Anopheles stephensi* mosquitoes. All 44 volunteers, including 13 who had not been immunized, developed parasitemia after challenge. Nevertheless, cellular immune responses and antibody responses were amplified significantly at 4 and 9 weeks after challenge, raising the possibility that DNA vaccination could accelerate the acquisition of naturally-acquired immunity in endemic areas and significantly reduce the morbidity and mortality associated with malaria infection.

Adenovirus 5 vectors with and without a DNA priming dose will be tested in phase 1/2a clinical trials soon (T. Richie, NMRC, personal communication). One vector will have an AMA-1 gene construct and the other vector will have a CSP antigen construct. These vectors have been shown to be immunogenic in mice and rabbits. The murine responses included antibody production and interferon-gamma ELISPOT responses. It is hoped that the adenovirus vector can in the future be used to carry multiple antigens .

A major project led by Adrian Hill and supported by the Bill and Melinda Gates Foundation Grand Challenges in Global Health is underway at Oxford University and collaborating institutions in the UK and USA to systematically test a variety of molecular adjuvants that will be co-expressed with antigen in plasmid DNA, poxvirus and adenovirus vectors in an effort to increase the potency of these vaccines for inducing cell-mediated and humoral immune responses. The adjuvants will be identified by screening cDNA libraries for molecules that induce phenotypes required to generate improved memory responses.

13. GENOMICS AND PROTEOMICS

The publication in 2002 of the complete genome sequence of *P. falciparum* strain 3D7 (195) and the rodent parasite *P. yoelii yoelii* (196) was a major advance in the understanding of malaria. The sequence of *P. falciparum* was predicted to contain 5268 genes. The online database plasmoDB, http://plasmodb.org/ set up by the National Institute of Allergy and Infectious Diseases, facilitates searching for *Plasmodium* gene sequences and curated annotations, and compiles all information associated with individual genes, such as predicted protein sequences and structures (reviewed in 197). A common strategy for making malaria vaccines is to incorporate antigens from different stages of the life cycle, so identification of stage-specific proteins of *Plasmodium* (8) is important. In addition, information on the variability of *Plasmodium* genes between and within strains helps to identify the molecules that are the subject of selective pressure, presumably due to the immune response.

The large number of known genes requires new screening methods to identify antigens that are likely to be useful in vaccines. Haddad and coworkers 2004 (198) have developed a high-throughput screening method based on identifying genes expressed during the sporozoite stage by comparing the annotated *P. y. yoelii* sequence with expressed sequence tags generated from a cDNA library of *P. yoelii* sporozoites. They immunized mice with single plasmids or pools of plasmids containing these genes, and then challenged mice with *P. yoelii yoelii*. From 192 genes tested, they identified five that reduced the parasitemia by at least 0.5 log, which represents a 68% to 79% decrease in parasite burden. The ultimate goal is to immunize humans with genes for homologous antigens from *P. falciparum*. Doolan and coworkers (199) developed algorithms to predict binding of peptides to HLA class I and class II supertypes. They analyzed peptide sequences generated by MudPIT (multidimensional protein identification technology, which combines in-line high-resolution liquid chromatography and tandem mass spectrometry) analysis of *P. falciparum* sporozoite preparations and scanned them against genomic sequences to identify open reading frames (ORFs) representing antigens potentially expressed in the sporozoite or liver stages of the life cycle. A set of 1,142 peptides was synthesized, representing those most likely to bind well to each HLA supertype. Peripheral blood mononuclear cells (PBMCs) were obtained from volunteers who had been

immunized with irradiated sporozoites and subsequently challenged with infectious sporozoites. Peptides were tested for their ability to stimulate these PBMCs to make interferon-gamma in an ELISPOT assay. The responses showed a trend for certain peptides to be recognized by protected as opposed to nonprotected volunteers.

Duffy and coworkers (200) are taking advantage of the knowledge gained about pregnancy-associated malaria to identify promising vaccine targets. They define the likely targets of protective immunity as proteins that are displayed on the infected erythrocyte surface of placental parasites, that react with the sera from multigravid women in endemic areas, elicit antibodies that cross-react with placental parasites from distant geographical areas, and adhere to chondroitin sulfate A or other placental receptors (the last criterion may not be fulfilled by all promising antigens). By limiting themselves to these proteins, they hope to target antigens that are expressed on parasites that are causing disease and are recognized by women who have protective immunity. They also note that genomic tools can identify polymorphic genes, which may reflect selection by the host immune system and therefore may be good vaccine candidates. These workers suggest that the best vaccine may require several antigens in order to target the variety of parasite forms that cause specific syndromes, and which have distinct patterns of gene expression. They also propose that the pathway they are using to assess candidate antigens for pregnancy malaria may be a paradigm for blood-stage malaria vaccine development.

14. INHIBITION AND EVASION OF THE IMMUNE RESPONSE BY MALARIA PARASITES

The inhibition and evasion of the immune response by malaria parasites has been reviewed by Urban and Roberts (201) and Hisaeda *et al.* (202). These processes help to explain why natural immunity is imperfect and why developing a vaccine is difficult. The fact that malaria parasites infect hepatocytes and red blood cells, and that the red blood cells can sequester in postcapillary venules, partly shields the parasites from an immune response. The parasites interfere with antigen presentation by their inhibitory effects on antigen-presenting cells and the production of peptides that interfere with presentation or elicit antibodies that block protective antibodies. Parasites elicit the formation of suppressive cytokines and cells, which affect immune responses that have been initiated despite the interference with presentation. Parasites respond to immune responses by changing the expression of surface antigens. It is not certain, but there is evidence that both the inhibition and the induction of apoptosis may play a role in evading the immune response. These mechanisms of immune evasion and suppression are described below. This section ends with a discussion of cryptic epitopes, which may be exploited to overcome one form of immune evasion.

14.1. Shielding

The first site of malarial parasite replication in humans is the liver, where it does not cause pathology (17), and which is tolerogenic for incoming antigens (reviewed in 203). The second site of replication is the red blood cell, which lacks MHC class I molecules and is therefore a poor target for the priming and effector functions of CD8+ T cells. The parasite within the red blood cell is shielded from the immune system by the membrane of the parasitophorous vacuole (the vacuole that forms upon invasion of a red blood cell or a hepatocyte and separates the parasite from the host cell cytoplasm) and the red blood cell plasma membrane.

14.2. Sequestration

The mature forms of the asexual blood stages sequester in postcapillary venules by cytoadherence. Sequestration ensures that at least a proportion of the infected red blood cells mature without passage through the spleen, where they would be removed by cordal macrophages due to their reduced deformability or opsonization with antibodies and/or complement (204-206).

14.3. Effects on antigen-presenting cells

The malaria pigment hemozoin paralyzes dendritic cells (207). Parasite-modulated dendritic cells fail to up-regulate the expression of major histocompatibilty complex (MHC) antigens or costimulatory or adhesion molecules and fail to secrete IL-12 and activate T cells, but secrete IL-10 and TNF-alpha in response to stimulation with lipopolysaccharide, CD40-ligand, TNF-alpha, or monocyte-conditioned medium (208). Urban and coworkers interpret these results as immunosuppression by the parasite.

When mice are immunized with irradiated sporozoites, Kupffer cells upregulate MHC class I antigens, costimulatory molecules, and the 40 kDa subunit of IL-12 (IL-12p40), and can present antigen, but in mice immunized with infectious sporozoites, the Kupffer cells down-regulate MHC class I antigens and the p40 subunit of IL-12, and as a consequence they have reduced antigen-presenting function (209).

When *P. falciparum* sporozoites are co-cultured with rat peritoneal macrophages, protein synthesis in the macrophages, as shown by the incorporation of tritiated leucine, is inhibited (210). The macrophages appear to undergo rapid degeneration. The authors propose that the inhibition of protein synthesis may explain why the immune response to natural infection by *Plasmodium* is generated slowly and is rarely, if ever, complete.

14.4 Altered peptide ligands

Malaria parasites can affect T cell priming in such a way as to suppress or evade the immune response. Plebanski and coworkers (211, 212) studied peptides from genetic variants of the *P. falciparum* circumsporozoite protein. When the cP26 and cP29 epitopes are presented together by antigen-presenting cells (APCs) to T cells, they abrogate the formation of cytotoxic T lymphocytes from naïve precursors to either epitope, a phenomenon called "immune interference". Such epitopes, which interfere with priming to other epitopes, are called "altered peptide ligands." In addition, a portion of the T cells primed to cP26 alone or cP29 alone preferentially recognize the other

epitope in restimulation assays. This is called "immune diversion."

14.5. Blocking antibodies

Guevara Patino and coworkers (213) found that antibodies to MSP-1_{83} (the NH_2-terminal 83 kDa domain of MSP-1) can block protective antibodies to MSP-1_{19}. They speculate that this may be a way for the parasite to evade an immune response that would block infection of red cells.

14.6. Immunosuppressive cytokines and cells

Macrophages in infected individuals suppress responses to blood-stage malaria (14) Soluble suppressive factors such as nitric oxide and prostaglandin E2 have been shown to be involved in suppression in some models, and transforming growth factor beta may also be involved (214). The effects include suppression of mitogen-induced T cell proliferation and cytokine production. Macrophages infected with *P. berghei* ANKA suppress the expression of the p40 subunit, but not the p35 subunit, of the complete IL-12 heterodimer (215). Inhibition of IL-12, a key cytokine in the induction of protective T helper type 1 immunity to blood-stage malaria, at the level of p40 gene expression or IL-12 p70 secretion, may be an important evasion strategy by the *Plasmodium* parasite (14). *P. falciparum* induces $CD4^+CD25^+$ regulatory T cells, *FOXP3* (a transcription regulator that is a marker of regulatory T cells) and TGF-beta that are associated with higher rates of parasite growth *in vivo* (216) .

14.7. Antigenic variation

There are many variant strains of parasites, so that people who are partially immune to one strain are often susceptible to another strain. Three families of variant genes have been characterized in *P. falciparum*. The best-studied are the *var* genes encoding *P. falciparum* erythrocyte membrane protein 1 (PfEMP1); the other two families are the repetitive interspersed family (*rif*) genes, and the subtelomeric variant open reading frame (*stevor*) genes (reviewed in 176). Even within an individual host infected with a single strain, an antibody response may kill many of the parasites, but those parasites that have switched *var* gene expression survive and multiply, producing a peak of parasitemia. As antibodies are made to the new *var* gene products, and as parasites switch again, sequential waves of parasitemia can develop (217). As another example of genetic variation that allows parasites to evade the immune response, some strains of *falciparum* can switch from a neuraminidase-dependent to a neuraminidase-independent invasion pathway (218).

14.8. Induction and inhibition of apoptosis

Protozoan parasites use both induction and inhibition of apoptosis to enhance their dissemination or survival within their hosts (reviewed in 219). The consequences of apoptosis of host cells in *Plasmodium* infections are complex and controversial. For example, Van de Sand and coworkers (220) found that the liver stage of *P. berghei* inhibits the apoptosis of the hepatocytes that they infect, while Leiriao and coworkers (221) showed that the amount of apoptosis of hepatocytes infected with untreated sporozoites is much lower than that of hepatocytes infected with irradiated sporozoites. Infectious sporozoites may inhibit the hepatocyte's intrinsic apoptosis pathway, while irradiated parasites cannot. Apoptosis of hepatocytes infected with irradiated parasites leads to recruitment of dendritic cells and phagocytosis of the hepatocytes containing parasite antigens. Therefore, the inhibition of apoptosis, which may result in the activation of parasite-specific CD8+ T cells, could represent a mechanism of immune evasion by normal sporozoites.

In contrast to hepatocytes, several studies have concluded that malaria parasites increase the amount of apoptosis of lymphocytes, though the consequences of this apoptosis are controversial. Helmby and coworkers (222) found an increase in the apoptosis of T cells, B cells, and macrophages in the spleens of *P. chabaudi chabaudi*-infected mice. Xu and coworkers (223) found an increase in the apoptosis of parasite-specific T cells in mice infected with lethal and nonlethal species of *Plasmodium*. They found that the deletion of parasite-specific T cells resulted in impairment of the ability of the mice to control parasitemia. On the other hand, Riccio and coworkers (224) found no significant correlation in humans between malaria-associated apoptosis of T cells and either parasitemia or the number of previous malaria attacks. Struik and Riley (26) propose that apoptosis of parasite-specific T cells represents a homeostatic control mechanism.

14.9. Cryptic epitopes

The use of cryptic epitopes to circumvent one type of immune evasion is discussed by Good (225). Cryptic epitopes are those peptides of a protein that are not recognized during an immune response to the protein for any of a variety of reasons, including incomplete or excessive processing or competition by other peptides (226). Epitopes that are immunodominant tend to be polymorphic, which suggests that parasites have developed mechanisms to evade immune responses. Cryptic epitopes are not polymorphic, because they are not the subject of selective pressure, but in some cases, T cells generated by immunization with cryptic peptides can react with the native protein. For example, Amante and coworkers (227) defined cryptic epitopes on *P. chabaudi adami* AMA-1. T cells specific for cryptic epitopes afforded partial protection against *P. c. adami* infection in athymic nude mice. This suggests that vaccines can be made that would circumvent the problem of antigenic variation. There is a potential problem in that responses to peptides are MHC restricted, so a given epitope may only be recognized by a fraction of the population. However, Krzych and Snyder (unpublished data) found that the cryptic epitopes on *P. berghei* CSP were nearly identical for three mouse strains that differ in MHC type.

15. POSSIBLE UNDESIRABLE EFFECTS OF VACCINATION

Gandon and coworkers (228) used mathematical modeling to determine the likely effect of vaccines on parasite evolution. They found that anti-growth rate and anti-toxin

vaccines would increase the virulence of the parasite population, because these vaccines remove the cost of virulence (increased mortality) without affecting its benefit (increased transmission), while anti-infection and transmission-blocking vaccines would reduce virulence. MacKinnon and Read (229) experimentally tested the model with *P. chabaudi* infection in mice. They found that serial passage of parasites through immunized mice resulted in an increase in virulence of the parasite population, as measured by the minimum red blood cell density over the following 18 days. Even if these concerns are justified, the problem may be avoided by developing a vaccine that includes antigens from several stages of the parasite life cycle.

Hoffman and Miller (230) pointed out that different vaccines may be required for different human populations. For example, vaccines that prevent infection are required for individuals who have never been or are infrequently exposed to malaria when they visit malarious areas, such as tourists, military personnel, foreign service workers, and business people. These authors warn that such vaccines may be dangerous for semi-immune people in malarious areas if the immunity is not life-long, because the vaccines would eliminate the natural boosting provided by low-grade infections. For these people, vaccines to reduce the parasite burden or neutralize the pathogenic properties of the parasites or the responses to them may allow the maintenance of immunity by continuous natural boosting. However, vaccines to prevent infection may be useful even for these people. An analogy can be drawn to insecticide-impregnated bednets. When infants in Burkina Faso were divided into two groups, those that were protected by bednets from birth and those that were protected by bednets beginning at age 6 months, there was a significantly lower incidence of malaria in the first group over the first 12 months of life, but no significant difference in the incidence of malaria during the follow up period of up to 3.5 years (231). This study suggests that protection against malaria in infants does not interfere with the development of protective immunity. Older people, who have already developed partial immunity, may also benefit from a pre-erythrocytic vaccine, because there is evidence that partial immunity to malaria may persist for several decades and that antibody titers to sporozoites are rapidly boosted following periods of increased malaria transmission (reviewed in 24). Even when antibody levels to sporozoites have declined to the point at which they are no longer protective on their own, they may reduce the parasite burden and allow time for the recall of blood-stage immunity.

Since many of the mechanisms that protect against malaria are also involved in the pathology of disease, vaccines must be carefully examined to be sure they do not cause harm. Artavanis-Tsakonas *et al.* (232) propose that a protective immune response to malaria involves a balance between pro-and anti-inflammatory cytokines. They point out that an inadequate production of proinflammatory cytokines will fail to eliminate the parasite, but the timely induction of anti-inflammatory cytokines is needed to control the immune response so that it does not cause immune-mediated pathology in the host. The authors conclude that this balance must be achieved for malaria vaccines to work properly. Perkins and coworkers (233) found evidence that this balance affects disease severity in children in Gabon, since low levels of IL-12 and transforming growth factor beta 1, and high levels of tumor necrosis factor-alpha and IL-10, were found in children with severe malaria. Hirunpicharat and coworkers (234) found that the same Th1 cells that protect against parasitemia can cause disease. Similarly, Hafalla and coworkers (235) found that CD8+ T cells may have a pathogenic role in murine cerebral malaria.

16. CASE DEFINITION, ACCURACY, AND EFFICACY

Rogers and coworkers (236) have identified a possible problem in the use of certain clinical case definitions in vaccine efficacy trials. For trials of pre-erythrocytic vaccines such as RTS,S in which the case definition is any parasitemia at all (49), the case definition is straightforward, but when the case definition includes a threshold parasitemia, which is common for trials of blood stage vaccines but also may be appropriate for trials of pre-erythrocytic vaccines (for example, the RTS,S trial of Alonso and coworkers (55)), the choice of a case definition is difficult. Rogers and coworkers (236) analyzed the results of three studies of malaria in children in Ghana, including 1488 outpatient episodes for which data on fever and parasitemia were available. They modeled a variety of case definitions that included fever and a threshold parasitemia. Within the range of parasitemia thresholds commonly used in interventional trials (1-5000 parasites/microliter) the measured efficacy of a vaccine varied from 21% to 79%, assuming that the vaccine reduced parasitemia by 90% at each observation. These workers propose replacing arbitrary thresholds with the use of logistic regression modeling to estimate the fraction of fevers attributable to malaria.

When the evaluation of clinical trials depends on parasitemia data, errors in microscopy can alter the results. Ohrt and coworkers (237) simulated a 12-week trial with weekly routine malaria smears. The readings of newly-hired microscopists had a sensitivity of 91% and a specificity of 71% as compared with those of expert microscopists. A very high specificity (>99%) for each malaria smear was found to be necessary for an estimate of protective efficacy to be within 10-25% of the true value. Using a statistical model that included various attack rates, the authors concluded that false positive results can have a profound effect in underestimating protective efficacy of a drug or vaccine, while false negative results do not significantly affect the estimate of protective efficacy. Ohrt and his coworkers have established a Malaria Diagnostics Centre for Excellence in Kisumu, Kenya, to train microscopists in proper methods of malaria diagnosis (C. Ohrt, WRAIR, personal communication, 2006).

17. CONCLUSION

Progress toward the development of a malaria vaccine has been disappointingly slow. The organism is far

more complex than the organisms targeted by most vaccines, and it is capable of evading and suppressing the immune response. The naturally-occurring immune response, which allows millions of parasitemic people to lead normal lives without symptoms, allows the parasite to continue to spread from person to mosquito to person. The sequencing of the *P. falciparum* genome, the development of new techniques to make subunit and whole-cell vaccines, and advances in our understanding of the immune response against malaria may allow the development of new and more successful vaccines against this old scourge.

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Abbreviations: AMA-1: apical membrane antigen-1, APC: antigen-presenting cell, CSP: circumsporozoite protein, ELISPOT: enzyme-linked immunospot assay, ELISA: enzyme-linked immunosorbent assay, FMP, *falciparum* malaria protein, GLURP: glutamate-rich protein, GSK: GlaxoSmithKline, HLA: human leukocyte antigens, IFA: immunofluorescence assay, IFN: interferon, LSA: LSA-1: liver-stage antigen-1, ME: multi-epitope, MHC: major histocompatibility complex, MSP-1: merozoite surface protein-1, MVA: modified vaccinia virus Ankara, NK: natural killer, PBMCs: Peripheral blood mononuclear cells, PfEMP1: *P. falciparum* erythrocyte membrane protein 1, Pfs: *P. falciparum* sexual-stage antigen, RESA: ring-infected erythrocyte surface antigen, RTS,S: vaccine based on circumsporozoite protein repeat region and T cell epitopes and hepatitis B surface antigen, TLR: toll-like receptor, TNF: tumor necrosis factor, TRAP: thrombospondin-related adhesive protein, WHO: World Health Organization, WRAIR, Walter Reed Army Institute of Research

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