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MINIREVIEW

Chemical Attenuation in the Development of a Whole-Organism Malaria Vaccine

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ABSTRACT Malaria vaccine development has been dominated by the subunit approach; however, many subunit vaccine candidates have had limited efficacy in settings of malaria endemicity. As our search for an efficacious malaria vaccine continues, the development of a whole-organism vaccine is now receiving much scrutiny. One strategy currently being explored in the development of a whole-organism vaccine involves chemical attenuation of the malaria parasite. *In vivo* and *in vitro* chemical attenuation of both liver-stage and blood-stage *Plasmodium* parasites has been investigated. Here, we discuss both approaches of chemical attenuation in the development of a whole-organism vaccine against malaria.

KEYWORDS chemical attenuation, malaria, whole-organism vaccine

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Current methods in malaria control, such as the use of indoor residual spraying, insecticide-treated nets, and drug therapies, have led to reductions in malaria cases and malaria-related deaths (1). However, malaria still remains a leading cause of morbidity and mortality, with 3.3 billion people at risk of becoming infected with *Plasmodium* parasites (1). In 2015 alone, there were 212 million clinical cases and approximately 429,000 deaths due to malaria (2); therefore, the need for an efficacious malaria vaccine remains prominent. However, despite concerted efforts over many years, an effective malaria vaccine remains elusive. There have been continued concerns with the efficacy of the subunit vaccine approach (3–6), leading to a renewed interest in the use of the whole-organism vaccine approach for malaria.

Whole-organism vaccines have been used successfully to protect individuals and populations from infectious diseases (reviewed in reference 7). This approach maximizes the number of antigens presented to the immune system and thus may limit the impact of antigenic diversity on vaccine efficacy. There are different types of whole-organism vaccines, including killed and live attenuated vaccines, that can be produced using heat, genetic manipulation, radiation, or chemicals. Some of the earliest vaccines utilized formalin to chemically treat the infectious organism (reviewed in reference 8). These vaccines are often referred to as "inactivated" vaccines and include vaccines against polio, hepatitis A, and cholera (9–11). More recently, chemicals have been used as attenuating agents in the development of a malaria vaccine, with promising results.

CHEMICAL ATTENUATION OF MALARIA PARASITES *IN VIVO*

Some of the earliest evidence that protection could be induced by infection and drug cure in malaria was provided by antimalaria drug evaluation studies in monkeys (12) and studies using malaria therapy in patients with neurosyphilis (13, 14). Although not the primary aim of these studies, they demonstrated that drug cure given after a patent primary infection induced protection against a subsequent secondary infection,

by reducing parasitemia and the severity of disease (12–14). Since these initial studies were conducted, numerous studies (in animals and humans) have focused on the protection and immunity induced by a regimen of infection and drug cure against subsequent parasite challenge (15–22). This regimen represents *in vivo* chemical attenuation of the parasite and, therefore, of the infection.

IN VIVO CHEMICAL ATTENUATION FOLLOWING INOCULATION OF SPOROZOITES

An initial study of *in vivo* chemical attenuation of malaria sporozoites used intravenous (i.v.) infection under chloroquine cover (15). Mice were infected five times with *Plasmodium berghei* sporozoites while receiving chloroquine treatment. Chloroquine targets blood-stage parasites but not pre-erythrocytic parasites, allowing the parasites to infect and develop within the liver before being killed at the trophozoite and schizont stages of the blood-stage life cycle (23). Protection was assessed by the survival of mice following homologous sporozoite challenge; all immunized mice survived, whereas all controls succumbed to infection (15). This study did not evaluate whether the protection was sterile (15); however, other studies have shown that a regimen of sporozoite infection under chloroquine cover can provide sterile protection against a subsequent sporozoite challenge (22, 24, 25). Mice immunized intravenously with one or two cycles of infection with *Plasmodium yoelii* sporozoites and drug cure had sterile protection (90% or 100%, respectively) following a sporozoite challenge infection (22). Protection was mediated by $CD4^+$ and $CD8^+$ T cells through the production of interferon gamma (IFN-γ) and nitric oxide, and although antibodies against pre-erythrocytic and blood-stage parasites were detected, they did not appear to play a role in the protective immune response (22).

A similar immunization strategy was used with the antimalaria drug mefloquine (26), which also kills blood-stage parasites (reviewed in reference 27). Mice immunized twice with live *P. yoelii* sporozoites while receiving mefloquine treatment had sterile protection against homologous sporozoite challenge, as well as a delay in patency and reduced peak parasitemia when challenged with sporozoites of a heterologous species (26). Immunized mice also had significantly reduced parasitemia compared to control mice, following direct i.v. challenge with homologous and heterologous blood-stage parasite species (26). Protection against blood-stage parasite challenge was also demonstrated in the chloroquine study (22), with all immunized mice showing lower peak parasitemia than control mice.

In these experiments, however, it is not clear whether there was genuine cross-stage protection or whether mice separately developed immunity to sporozoites and blood-stage parasites as a result of exposure to both.

Sterile protection has also been demonstrated in human volunteers who received a regimen of three inoculations of sporozoites (by mosquito bite) under chloroquine cover (17, 28). Following immunization, antibody responses to both sporozoites and blood-stage parasites and to recombinant parasite proteins, as well as a significant increase in the proportion of cells producing multiple cytokines (IFN-γ, tumor necrosis factor alpha [TNF-α], and interleukin-2 [IL-2]) in response to *Plasmodium*-infected red blood cells (17), developed in recipients. Protection following challenge infection was associated with pluripotent effector memory T cells (17, 29). Sterile protection was long lasting, with 4 of 6 immune volunteers remaining aparasitemic following rechallenge 28 months after the initial challenge infection (29). The two remaining immunized volunteers had delayed patent parasitemia (29). IFN-γ production following *in vitro* stimulation of peripheral blood mononuclear cells from immunized individuals with cryopreserved sporozoites or *Plasmodium falciparum*-infected red blood cells was detectable for up to 14 months following this infection and drug cure regimen (30). Both $\alpha\beta$ and $\gamma\delta$ T cells with effector memory phenotypes were associated with this response (30). The protection induced was mediated against pre-erythrocytic stage parasites, as there was no evidence of protection following direct i.v. blood-stage challenge (28). In these experiments, however, the parasitemia was followed to a level of approximately

10,000 parasites per ml of blood. It is not known whether blood-stage protection would be observed at a later time in the infection (as in the mouse experiments described above). Additionally, this immunization regimen provided partial protection against sporozoite challenge with a heterologous strain, with sterile protection observed in 2 of 11 volunteers and the remaining volunteers having a delay in patency (31).

Administration of cryopreserved, infectious *P. falciparum* sporozoites (Sanaria PfSPZ Challenge) to malaria-naive volunteers receiving chloroquine chemoprophylaxis has also been assessed (32, 33). In the initial study, volunteers who received either three or four intradermal (i.d.) injections of sporozoites at monthly intervals while taking chloroquine had limited immune responses and were not sterilely protected following sporozoite challenge by mosquito bites (32). However, the second study, which used three intravenously administered doses of 5.12×10^4 sporozoites at 28-day intervals while volunteers were receiving cholorquine, provided 100% sterile protection following a controlled human malaria infection (33). Protection was associated with parasite-specific polyfunctional $CD4^+$ memory T cells.

Another immunization strategy used isopentaquine, which can attenuate the infection at the liver stage, followed by sporozoite infection (34). C57BL/6 mice were given chemoprophylaxis for 3 days before direct i.v. infection with *P. berghei* ANKA sporozoites. Immunized mice had a delay in blood-stage parasite patency compared to control mice and did not suffer from experimental cerebral malaria (ECM) (34), an outcome which is usually associated with this model. This regimen induced an earlier than normal proinflammatory immune response, with activation of $CD4^+$ and $CD8^+$ T cells in the liver and of $CD8^+$ T cells in the spleen, as well as increased production of IFN-γ, TNF-α, and IL-2, which was protective against ECM (34). However, immunized mice eventually succumbed to infection due to hyperparasitemia-associated anemia (34).

IN VIVO CHEMICAL ATTENUATION FOLLOWING INOCULATION OF BLOOD-STAGE PARASITES

Numerous studies comprising *in vivo* chemical attenuation of blood-stage infections have been conducted in rodents (16, 20, 21, 35). Furthermore, immunity in both humans and rodents was observed following administration of multiple low doses of blood-stage parasites with each infection treated prior to patency (19, 20). In the rodent model, mice infected three times with blood-stage *P. chabaudi* parasites followed by drug cure with atovaquone-proguanil (48 h later) were protected against subsequent blood-stage challenge with homologous and heterologous parasite strains (20). This immunization regimen induced antibody and cellular immune responses to both homologous and heterologous parasite strains, suggesting that the antigenic targets of immunity were conserved (20). These subpatent infections induced protective T cells (20). Other studies in rodents have shown that exposure to blood-stage parasites under chloroquine cover from the outset can induce cross-stage protection against sporozoite challenge (16, 21). Administration of one or two doses of *P. yoelii*-infected red blood cells under chloroquine cover conferred long-lasting protection against blood- and liver-stage parasites in mice. Both $CD4^+$ and $CD8^+$ T cells were required to inhibit development of liver-stage parasites, through the action of nitric oxide and IFN-γ (16). Antibodies against both pre-erythrocytic and blood-stage parasites were also induced and were essential for protection (16).

In humans, repeated submicroscopic *P. falciparum* blood-stage infections followed by drug cure with atovaquone-proguanil protected individuals from a further submicroscopic infection (19). Protection was associated with a strong cell-mediated immune response involving both $CD4^+$ and $CD8^+$ T cells, as well as the production of IFN-γ (19). Nitric oxide synthase levels were also increased in peripheral blood mononuclear cells of immune individuals. However, a possible contribution of residual atovaquone to this observed protection could not be ruled out, due to the lengthy persistence of this drug (36).

CHEMICAL ATTENUATION OF MALARIA PARASITES *IN VITRO*

The method of *in vitro* chemical attenuation of *Plasmodium* parasites was initially investigated using sporozoites in the 1980s (25). *P. berghei* sporozoites were incubated in the presence of high concentrations of chloroquine and were rendered noninfective, as determined by the absence of patent parasitemia following immunization. Mice received five doses of these chloroquine-attenuated parasites and were protected following challenge with wild-type sporozoites (25). To our knowledge, however, this strategy using chloroquine has not been pursued and the mechanism of action of chloroquine against sporozoites has not been defined.

More recently, DNA-binding drugs have been used for the *in vitro* attenuation of *Plasmodium* spp. Centanamycin (CM) and tafuramycin-A (TF-A) belong to a family of chemical agents that irreversibly bind to poly(A) regions of DNA (37), which are abundant in the A-T rich genome of *Plasmodium* spp. (38, 39). Immunization of mice with either *P. berghei* or *P. yoelii* CM-attenuated sporozoites resulted in the induction of antigen-specific IFN-γ-producing $CD8^+$ T cells and high titers of antibodies to whole sporozoites. A multiple-dose regimen induced sterile protection against homologous and, in the case of immunization with chemically attenuated *P. berghei*, heterologous species challenge (40, 41).

In vitro chemical attenuation of blood-stage parasites has also been investigated using CM and TF-A (42, 43). Immunization with either a single dose of *P. chabaudi* or three doses of *P. yoelii* chemically attenuated blood-stage parasites resulted in activation of both $CD4^+$ and $CD8^+$ T cells, and strong proliferative responses were induced against both homologous and heterologous parasites (42, 43). Inoculation of chemically attenuated blood-stage *P. yoelii* parasites also induced parasite-specific antibodies (43). Mice were protected against homologous blood-stage parasite challenge, with protection being mediated by $CD4^+$ T cells (42, 43) and B cells (in the case of the *P. yoelii* vaccine) (43). Immunization with chemically attenuated blood-stage parasites can also provide protection against challenge with heterologous blood-stage parasite strains (42, 43) and species (42). The protection induced by chemically attenuated *P. yoelii* parasites was stage specific, as immunized mice were not protected against i.v. or mosquito bite sporozoite challenge (43). However, there was a significant reduction in peak parasitemia compared to control mice when the sporozoite-initiated infection was followed through to the blood stage (43).

Further preclinical studies have been conducted using chemically attenuated blood-stage *P. falciparum* strain FVO parasites administered to *Aotus nancymaae* monkeys (44). A proliferative $CD8^+$ T cell response was induced following immunization with a single dose of TF-A-attenuated *P. falciparum*. Although only limited protection was seen in this model (44), further vaccine doses may improve protection.

A pilot study has been conducted in human volunteers to assess the safety and immunogenicity of *P. falciparum* blood-stage parasites attenuated *in vitro* with TF-A (reviewed in reference 45). Further studies are required to evaluate the protective efficacy of these *in vitro* chemically attenuated *P. falciparum* blood-stage parasites in humans.

CHALLENGES OF THE CHEMICALLY ATTENUATED VACCINE APPROACH

The development of a chemically attenuated malaria vaccine has its challenges. A prominent challenge for the use of *in vivo* chemical attenuation, with its multiple cycles of infection and drug treatment, is whether it can be used as a widely implementable vaccine strategy in areas of malaria endemicity. There are also a number of challenges with the use of *in vitro* chemically attenuated parasites, such as underattenuation of parasites and the use of human blood and blood products, as well as the storage and deployment of such a vaccine (Table 1). However, progress toward addressing these issues is being made through rigorous testing and methods of cryopreservation.

TABLE 1 Challenges in developing malaria vaccines based on *in vitro* chemical attenuation

Challenge	Solution
Underattenuation of parasites	*In vitro* assessment and preclinical studies can provide preliminary data on the amount of the chemical required to adequately attenuate the parasite. However, this needs to be confirmed by rigorous assessment in pilot studies and phase I safety trials in humans.
Use of human blood in chemically attenuated blood-stage vaccines and the possible induction of antibodies against red blood cell surface antigens (ABO and Rh blood group systems)	This can be greatly limited by the use of red blood cells from universal donors (blood group O Rh negative). Additionally, antibodies to minor antigens can be addressed by limiting the number of uninfected red cells in the vaccine.
Storage and deployment of vaccine	Cryopreservation of the vaccine is likely to be required. Methods for cryopreserving unattenuated sporozoites and blood-stage parasites, as well as irradiation-attenuated sporozoites, have been established (46, 47). These methods may be applicable to the cryopreservation of *in vitro* chemically attenuated sporozoites and blood-stage parasites.

CONCLUSION

An efficacious vaccine will greatly aid efforts to eliminate and, eventually, eradicate malaria. Such a vaccine will need to provide long-lasting strain-transcending protection. The studies presented in this review provide evidence that immunization with malaria parasites, whether attenuated *in vivo* or *in vitro*, can induce protective parasite-specific immune responses. The protection provided can be both long lasting (30, 43) and strain and species transcending (20, 31, 42, 43). Chemically attenuated blood-stage vaccines may currently be difficult to implement in settings of malaria endemicity; however, novel strategies for cryopreservation and drug delivery are likely to reduce the barriers to these approaches in the near future.

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