

REVIEW

Malaria: An Evaluation of the Current State of Research on Pathogenesis and Antimalarial Drugs

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This brief review provides an overview of the pathogenesis of malaria, the human immune response to malaria, current methodology for malaria diagnosis, and current antimalarial drug regimens. The review also provides a critical evaluation of the research directions in the areas of drug design and vaccine design.

INTRODUCTION

Malaria is inarguably the world's most devastating disease. It is endemic in sub-Saharan Africa, South Asia, and parts of South America, and it kills more than 800,000 people every year [1]. It is the leading cause of death in children and directly and adversely affects economic development. In spite of its defined pathogenesis and a number of available treatments, the disease remains a huge economic burden. This review will provide a broad evaluation of the state of research on the pathogenesis of and immune responses to malaria as well as the efficacy of the cur-

rent repertoire of antimalarial drugs and the best approaches for the development of new antimalarial drugs.

PATHOGENESIS OF MALARIA

Malaria can be caused by any of five species of the protist genus *Plasmodium* — *P. vivax*, *P. ovale*, *P. malariae*, *P. falciparum*, and *P. knowlesi*. *Plasmodium* depends on two hosts — a female mosquito and a human — to complete its life cycle.

The female *Anopheles* mosquito bites an infected host, and gametocytes of the parasite are released in the mosquito gut.

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†Abbreviations: WHO, World Health Organization; RDTs, rapid diagnostic tests; ELISAs, enzyme-linked immunosorbent assays; PCRs, polymerase chain reactions; LAMP, loop-mediated isothermal amplification; ACTs, artemisinin-combination therapies; DTT, dichlorodiphenyltrichloroethane; TBV, transmission-blocking vaccine; AnAPN-1, *Anopheles alanyl aminopeptidase 1*.

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These gametocytes mature into gametes, which then form diploid zygotes that burrow into the midgut epithelium and develop into oocysts. The oocysts burst after one or two weeks of incubation in the midgut and release sporozoites into the hemolymph. These sporozoites invade the salivary glands and are then transmitted to the bloodstream of the next human that the mosquito bites.

In the human host, the sporozoites migrate to the liver and infect hepatocytes, using these host cells to grow and form haploid merozoites. This stage can take less than a week, but often the parasites lie dormant in the liver, leading to disease relapses weeks or months following infection. The merozoites exit the hepatocytes and infect erythrocytes, using them as sites of asexual replication by remodeling the erythrocytes. New merozoites burst from the erythrocytes and infect more cells, leading to the classic symptoms of malaria — paroxysms defined by intense chills, fever, and sweating. Some merozoites mature into gametocytes outside of erythrocytes and are then taken up by another mosquito, leading to a repeat of this entire life cycle [2,3]. Malaria also can be transmitted via blood transfusions and organ transplants as well as congenitally from a mother to her fetus.

There are some interspecies differences for the *Plasmodium* life cycle. The frequency of paroxysms can vary depending on the speed of asexual replication and erythrocyte bursting. *P. vivax*, *P. malariae*, and *P. ovale* can all cause relapses; specifically, *P. malariae* can persist for decades before manifesting any symptoms [4]. While all species infect erythrocytes, only *P. falciparum* can infect erythrocytes in any stage of erythrocyte development [5]. *P. falciparum* infections can thus lead to an entirely infected bloodstream and far more frequent paroxysms than for infections with other *Plasmodium* species.

THE HUMAN IMMUNE RESPONSE TO MALARIA

The classic symptoms of malaria are paroxysms defined by intense chills, fever, and sweating. Chills will often be accompanied by headaches, nausea, and fatigue. Of

course, these symptoms are common to many illnesses, and a definitive diagnosis requires additional clinical tests and/or follow-up questions, particularly for patients in non-endemic regions. In severe cases, patients may present with respiratory and neurological problems as well as severe anemia and renal failure. Cerebral malaria is a rare complication; it primarily affects young children, and its cause is unclear. Placental malaria is a significant complication in endemic areas, but like cerebral malaria, its induction is poorly understood, although gene polymorphisms in the fetus have been found to affect the severity of placental malaria [6]. Our poor understanding of these disease outcomes has much to do with an incomplete knowledge of the host immune response to malaria and immune-evasion tactics of the parasite. However, it also exposes a general lack of knowledge about placental and cerebral immunity. Research on these areas should be actively encouraged.

There is little data on the human immune response to malaria, such as cytokine profiles for various strains of *Plasmodium* and various disease severities, what *Plasmodium* antigens are activating the innate immune system, what innate immune receptors are most relevant, and how *Plasmodium* evades immune system detection [6]. The adaptive immune response is also poorly understood. Both B cells and T cells are important for *Plasmodium* clearance. The *Plasmodium* epitopes and antigen-derived peptides that B cells and T cells respectively recognize have not been well characterized. Due to the chronic nature of some *Plasmodium* strains, it is suspected that both T cells and B cells may be exhausted (and less functional) in these patients [6]. Interestingly, a recent study found that most patients have functional memory B cells and long-lived antibody titers (although protectiveness could not be assayed) [7]. Further research is required, but this suggests that vaccines focused on activating B cells could be successful. Additionally, depletion of regulatory T cells (which dampen immune responses) in mouse models of malaria enhances the immune response to malaria [6]. Regulatory

T cell activity may thus be a good target for drug modulation.

There are several challenges in developing a viable, broadly effective human vaccine to malaria. First, the most vulnerable populations (children, pregnant women, and immunocompromised individuals) also may be unable to receive the vaccine or develop protective immunity from the vaccine without booster shots. As a result, the cost of the vaccine could be prohibitive. Second, malaria is endemic in regions of Africa that are already struggling with tuberculosis and HIV infections. Co-infections make the prospect of eliminating the parasite more difficult. Third, *Plasmodium* has several life stages, making selection of important antigens for targeting in a vaccine more challenging. Fourth, a vaccine should both reduce disease on an individual level and also lower the transmission rate within the population; depending on what antigens are targeted, both of these goals may not be achieved. Finally, as indicated above, we have a very poor understanding of the immune response to malaria. Nonetheless, development of human vaccines to malaria is ongoing. There have been no successful blood stage vaccines, although use of attenuated *Plasmodium* deficient in essential proteins for merozoite have shown some promise in mouse models of malaria [3]. The pre-erythrocytic stage may be more promising for vaccine targeting; it is known that immunization with irradiated sporozoites, which can infect the liver but not undergo the liver-stage of development, induces immunological memory [3]. As with the erythrocyte stage, attenuated *Plasmodium* strains may be effective. Additionally, subunit vaccines using immunodominant epitopes also could be effective. However, for vaccine development to progress efficiently, it is vital to close the numerous, large gaps in our understanding of the human immune response to malaria.

METHODS FOR DIAGNOSING MALARIA

The simplest diagnostic test is to stain a sample of the patient's blood with a Romanovsky stain (a broad term for eosin-meth-

ylene blue stains) and then examine it microscopically. If the patient is infected with malaria, *Plasmodia* will be visible inside erythrocytes. This method is effective, particularly in impoverished areas, which lack access to more sophisticated diagnosis methods. However, slide examination can be time-consuming and somewhat subjective; additionally, detection limits can mean that appropriate therapeutics are withheld for the patient until it is too late [8]. Current data from the World Health Organization (WHO) suggests that the quality and accuracy of slide examinations is generally poor in Africa [9].

Another diagnostic method is rapid diagnostic tests (RDTs), in which the patient's blood is applied to plates or pads containing antibodies specific for malarial antigens. If a series of bands is visible after a short amount of time and match bands given by a positive control sample, the patient has malaria. Both RDTs and microscopy can be used to determine the specific *Plasmodium* species the patient harbors, but positive RDTs are typically confirmed with microscopy, as false-positive RDTs are relatively common [10].

Enzyme-linked immunosorbent assays (ELISAs) and polymerase chain reactions (PCRs) also can be used to determine infection and/or past exposure to malaria, but these techniques are substantially slower and therefore are not practical for use in the healthcare system. However, a recent PCR-alternative has shown some promise in early testing. Loop-mediated isothermal amplification (LAMP) is similar to the PCR method, but it does not depend on temperature changes to mediate amplification. Therefore, LAMP does not require PCR blocks, which are too expensive for impoverished areas where malaria is endemic, and it is faster than traditional PCR [8]. Additionally, LAMP can be modified to allow for visual detection similar to that in an ELISA or RDT. Primer sets have been developed that are specific for *P. falciparum* and for all species of *Plasmodium*, respectively. LAMP, using these primer sets, was demonstrated to accurately identify malaria infections in all low-parasite patient isolates tested [8]. This method seems to combine the advantages of

both RDTs and microscopy, while also allowing for earlier diagnoses when parasite levels are low and, therefore, more controllable with drug treatments. Since primers can be easily adjusted and are generally functional at the one or two base pair mutation level, LAMP could be a robust diagnostic tool even in areas with rapid emergence of drug-resistant malaria strains. LAMP will still be relatively expensive in comparison to microscopy, but over time may prove to be more a cost-effective option if earlier diagnoses can indeed be consistently achieved.

EVALUATION OF CURRENT ANTIMALARIAL DRUGS

There are several classes of drugs used to treat malaria. All of these drugs target the intraerythrocytic stage of the parasite life cycle, when symptoms are first detectable. Antimalarial drugs include quinolines, antifolates, and artemisinin-combination therapies (ACTs).

Quinolines — quinine, chloroquine, mefloquine, and amodiaquine — are basic aromatic compounds. They are the oldest class of antimalarial drugs. Quinine, isolated from cinchona tree bark, has been used to treat malaria since the early 17th century. Its mechanism of action has not been deduced, but it has been inferred through research on other quinolines. All quinolines are thought to function as hemozoin inhibitors. Hemozoin is a byproduct of hemoglobin proteolysis in the *Plasmodium* food vacuole. When inside erythrocytes, the parasite depends on hemoglobin proteolysis for nutrition. Heme itself is toxic to *Plasmodium*, and the parasite must therefore convert it to a non-toxic form. The mechanism for this conversion is unclear — neither humans nor mosquitoes produce hemozoin — but this process is, retrospectively, a good drug target. It has been shown *in vitro* that chloroquine binds to purified heme and associates with heme-containing fractions from *Plasmodium*, probably competing out free heme and, thus, inhibiting the conversion of heme to hemozoin [11]. Therefore, quinoline-resistant strains can be inferred to have mutations in genes encoding

proteins necessary for transport of the drug into the food vacuole of *Plasmodium*, and there are at least two documented vacuole membrane transporters that are mutated in chloroquine-resistant strains [12].

Antifolates include sulfadoxine and proguanil. These drugs block steps in the folic acid synthesis pathway, which is essential to *Plasmodium* growth because the parasite is unable to utilize pyrimidines already synthesized by the host and must use this pathway to make its own [12]. Antifolate resistant *Plasmodium* strains contain mutations in dihydrofolate reductase and dihydropteroate synthetase, targets of proguanil and sulfadoxine respectively [12,13]. These mutations reduce the binding affinity of the drugs to their targets while still enabling the parasite to manipulate the folic acid synthesis pathway for its own benefit.

ACTs are the cornerstone of current malaria treatments. Artemisinins are derivatives of the Chinese herb *Artemisia annua* that have been used since the late 1970s [14]. They are sesquiterpene lactones that reduce the parasite load substantially earlier than other antimalarial drugs; additionally, they can kill *Plasmodium* gametes and thus lower transmission rates [13]. Their mechanism of action is unclear, but it is speculated that, like quinolines, these drugs block the conversion of heme to hemozoin [13]. Although highly effective, artemisinins are recommended for use in combination with the more long-lasting quinolines or antifolates due to their short half-life *in vivo* in order to ensure complete elimination of residual parasites. As with other antimalarials, artemisinin-resistant strains of *Plasmodium* have already emerged, but ACTs enhance the chance that these resistant strains can be contained. WHO currently recommends ACTs as the primary treatment for most strains of malaria and for most patients with the exception of pregnant women in their first trimester [9].

METHODOLOGY FOR ANTIMALARIAL DRUG DEVELOPMENT

Good antimalarial drugs should kill *Plasmodium* in the erythrocyte infection stage

and should be active against drug-resistant strains [15]. Ideally, these drugs should be easy to manufacture and safe enough for vulnerable populations such as children and pregnant women. There are currently two major approaches to identifying antimalarial drug candidates. In the older “whole parasite” screening approach, chemical libraries are created and then used to treat erythrocytes cultured with *Plasmodium* [16]. Promising drug candidates are further screened for resistance before proceeding to clinical trials. The relatively recent completion of the human genome sequence has enabled a second “rational design” screening approach. Here, molecular targets — such as enzymes or important receptors — in *Plasmodium* are identified, validated, and expressed before screening with drug candidates. Since the mechanisms of action for many antimalarial drugs — and, therefore, the mechanisms of resistance against these same drugs — are very poorly understood, this latter approach is appealing. However, “rational design” screens appear to be less cost-effective than “whole parasite” screens. Given the fast evolution of *Plasmodium* and the slow process for drug development, testing, and approval, it is best that new antimalarial drugs are multi-target and the “whole parasite” screening approach facilitates this.

Ideally, both screening approaches can be used in tandem — the “whole parasite” screening approach for quick generation of drug candidates that can then be accelerated into clinical trials. During this phase, the drug could be returned to “rational design” screens for further identification of a mechanism of action. Alternatively, the drug could be re-evaluated for a mechanism of action in the “whole parasite” screening approach by culturing with increasing doses to apply selective pressure on *Plasmodium* and then sequencing the genomes of parasites that were drug-resistant to identify the gene mutations involved in mediating resistance. The mechanism of action and potential side effects of the drug could then be confirmed in *P. berghei* or *P. yoelii* infections of wild-type and knock-out mice.

The value of the “whole parasite” screening approach was recently highlighted

by the discovery of a new class of antimalarial drugs — spiroindolones — that are promising in a number of respects: effective at low concentrations, easy to manufacture and formulate, and with a clear mechanism of action [15]. The optimized spiroindolone, NITD609, has been tested in the *P. berghei* mouse model of malaria and was found to be effective at doses lower than those used for several other antimalarial drugs. By using increasing doses of NITD609 in culture, the researchers were able to identify the gene mutation involved in mediating resistance to NITD609 and, thus, the mechanism of action for NITD609 [15]. If NITD609 continues to be viable in clinical trials, this information will be highly useful.

COMBATING MALARIA AT THE LEVEL OF THE MOSQUITO

Recommended preventive measures against malaria are primarily focused on minimizing mosquito bites by following clothing guidelines and using insecticides and insecticide-treated bed nets. These methods, along with preventive care during pregnancy, are estimated to have saved almost three quarters of a million children in Africa over the last 10 years [17]. Increased investment in preventive care will save even more lives.

Pyrethroids are the most commonly used class of insecticides; however, many species of *Anopheles* are now known to be resistant. One long-standing controversy in preventive care has been the use of the pesticide dichlorodiphenyltrichloroethane, more commonly known as DDT. DDT has been used as a pesticide and antimalarial since the 1940s but was found to build up in apex predators leading to the near extinction of several bird species in the United States. Due to its persistence in the environment and its high toxicity, it was banned in the United States in 1972, but in 2006, the WHO once again endorsed its use for malaria prevention. A recent panel of environmentalists and epidemiologists also recommended DDT with the caveat that it should be a last-resort insecticide if safer alternatives are available

[18]. It is generally sprayed inside homes, but there have been few studies on the implications of this for human health. The panel evaluated hundreds of studies to conclude that DDT may adversely affect fertility and brain development in children as well as increase the risks for certain cancers and diabetes [18]. However, DDT is the most effective insecticide in many regions of Africa. The panel's recommendations are sound; DDT remains poorly studied, particularly in Africa, and it is vital that constant monitoring takes place in areas where households are being constantly sprayed. It also seems inevitable that, as with pyrethroids, DDT-resistant strains of *Anopheles* eventually will emerge and research should continue to focus on the development of new, minimally toxic drugs.

The controlled eradication of *Anopheles* is an appealing idea for the prevention of malaria and other mosquito-borne diseases, but both its impact on the broader ecosystem has been a concern. Interestingly, in a recent *Nature* article that queried many mosquito biologists and ecologists, the scientists were confident that the loss of the mosquito would not adversely affect the ecosystem in the long-term, because, somewhat surprisingly, mosquitoes are dispensable and/or easily replaceable for most of the ecological processes they participate in [19]. Even if mosquitoes cannot be eradicated, they are a good vaccination target. In January 2009, the PATH Malaria Vaccine Initiative announced a collaboration with the Sabin Vaccine Institute and the Johns Hopkins Bloomberg School of Public Health to promote the development of a transmission-blocking vaccine (TBV). TBVs would be administered to humans and contain key antigens vital for malaria growth in and transmission from mosquitoes. If these antigens can induce a sufficiently potent, targeted antibody response, the antibodies would be taken up by mosquitoes during the biting process and block further spread of malaria. Earlier TBVs that have used malaria-specific antigens have not been successful in clinical trials, but one recently identified antigen, *Anopheline* alanyl aminopeptidase I (AnAPN-1), appears quite

promising. AnAPN-1 is crucial for *Plasmodium* invasion of the *Anopheles* midgut and has had a high success rate in blocking both *P. vivax* and *P. falciparum* development in mosquitoes in initial trials [20].

Aside from malaria-specific and *Anopheles*-specific proteins involved directly in malaria transmission and development in the mosquito, other good candidates for TBVs could include proteins or stimuli that could enhance the development of "innate immune memory" in the mosquitoes, preventing future infection by the parasite. "Innate immune memory" is likely a misnomer, but long-lasting innate immune responses were recently found in *Anopheles gambiae* mosquitoes [21]. Here, mosquitoes challenged with *P. berghei* had lower numbers of oocysts in the midgut and increased numbers of granulocytes that also were morphologically different from those in naïve mosquitoes, and, intriguingly, cell-free hemolymph isolated from primed mosquitoes was protective against *Plasmodium* when transferred into naïve mosquitoes [21]. If the molecules that mediate the effects of primed hemolymph transfer — increased circulating granulocytes and oenocytoids and decreased prohemocytes — could be identified, they could form the basis for a new TBV or be incorporated into a pre-existing TBV.

CONCLUSIONS AND OUTLOOK

We are gaining ground in the battle with both the mosquito and the *Plasmodium* parasite, but we can do better. Preventive measures to minimize mosquito bites should continue to be the primary focus, but there should be increased investment in vaccine development. Unfortunately, our poor understanding of the immune response to malaria is an impediment to generating good vaccines. In order for vaccine development to lead to viable vaccines, it is crucial that we collect more data on immune system activity in response to malaria, thereby potentially identifying good targets for vaccine and drug treatment. Work should continue on TBVs and methods for combating *Plasmodium* within the mosquito. These mos-

quito-intrinsic efforts may prove more valuable in terms of reducing transmission rates.

The long-term efficacy of antimalarials always will be limited due to the fast emergence of drug-resistant *Plasmodium* strains. Optimal drug choice should be tailored to specific regions. As researchers have observed, the most cost-effective treatments for malaria may vary depending on antimalarial drug resistance in a given region. Additionally, a streamlined antimalarial drug design approach would make the generation of new drugs more efficient. Recent advances suggest that the “whole parasite” screening approach is faster than the “rational design” screening approach. Time is of the essence as drug resistance to *Plasmodium* species emerges very fast. Faster drug design does not mean that we should abandon attempts to understand the mechanisms of action of these drugs, but mechanistic studies are perhaps best conducted concurrently to the clinical trial phase of drug design.

The human and economic costs of malaria are sometimes forgotten by those of us fortunate to live in non-endemic regions where malaria was eradicated long ago. Numerous international and local groups are working tirelessly to provide cohesive guidelines for diagnosis and treatment. Progress may be slow, but slight shifts in funding and research priorities could finally give us the upper-hand against malaria.

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