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Design of a New Whole Merozoite Vaccine for Malaria

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Abstract. Malaria is one of the most fatal diseases in the sub-Saharan area, causing more than 200 million people infected and more than 600000 people dead every year. The whole society are working together to develop vaccine to prevent people from being infected. This research will focus on a new whole merozoites vaccine which contains CRISPR-Cas9 based CyRPA protein deficient merozoites. The vaccine is designed to prevent the merozoites from entering the red blood cells so it can stay in the blood, be exposed the immune system and stimulate immune response. This new vaccine can provide a durable and effective prophylactic effect if successful. The vaccine is expected to have no serious and fatal side effects and will undergo animal tests and voluntary tests to test its effectiveness. Further research will focus on resolve the remaining challenges of the vaccine, including red blood cell deformation and the possibility of merozoites still entering the blood cells.

Keywords: Malaria; Vaccine; Application; Security assessment; Efficiency analysis.

1. Introduction

Malaria is a parasitic disease caused by infection with malaria parasites, mainly through the bites of Anopheles mosquitoes carrying the parasite, blood transmission and mother-to-child transmission. Susceptibility to malaria is widespread, especially in malaria-endemic areas such as Africa and Southeast Asia, and young children, pregnant women, the elderly people, people with compromised immunity, and non-immunized people who have traveled to malaria-endemic areas are more susceptible. The main symptoms of malaria are chills, high fever, sweating, headache, fatigue and vomiting. After a certain incubation period, the symptoms will recirculate. In severe cases, malaria can lead to anemia, coma, acute renal failure and death. There are 4 species malaria parasites, including Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae and Plasmodium ovale. Plasmodium falciparum can cause the most morbidity and mortality in the 4 species malaria parasite. The sub-Saharan African region has the highest share of the global malaria burden, accounting for 94% t of malaria cases (233 million) and 95% of malaria deaths (580,000), with children under five years of age accounting for approximately 80 percent of total malaria deaths in the region.

Malaria parasite has 2 hosts, including human and Anopheles mosquito. It has different reproduction mode in human and mosquito, which asexual in human and sexual in mosquito. In human body, there are 2 cycle, including exo-erythrocytic cycle and erythrocytic cycle. In the exo-erythrocytic cycle, the sporozoites infect the human by the bite of infected female Anopheles mosquitoes, after sporozoites enter the blood, it will travel through the blood to the liver, and invade liver cell. Sporozoites develop and form schizonts in liver cell in next 5-15 days, Plasmodium falciparum do not have the dormant stage in liver cell, and it cannot cause relapses of malaria infection [1]. Each schizont contains 10,000 to 30,000 merozoites, merozoites are released into the blood circulation and invade the red blood cells, and then it is the erythrocytic cycle. In the red blood cell, the merozoite develops through ring, trophozoite and schizont stages. At least, schizonts break red blood cells, and release new merozoites, which invades new red blood cells. And repeat this process. The invasion by merozoites requires the interactions of specific receptors between erythrocyte receptors and merozoite ligands [2]. In this process, a small part parasite differentiates from merozoites into sexual forms,

which are macrogametocyte (female) and microgametocyte (male). When the female Anopheles mosquito bite the blood of the infected human, female gametes and male gamete enter Anopheles mosquito, and start sexual reproduction. The mature macrogametocytes travel with the blood into the midgut of Anopheles mosquitoes and escape from the red blood cells to form macrogametes (female gamete). And microgametocytes form eight haploid motile microgametes (male gamete) in the midgut of mosquito. Male gametes move quickly to fertilize female gametes and form zygotes. The non-motile zygote transforms into motile ookinete in 18-24 hours. Ookinetes cross the midgut epithelium, and reach the extracellular space between the midgut epithelium and the overlaying basal lamina. In 10 to 24 days after infection, oocysts release thousands of sporozoites into blood, which invade the salivary gland of Anopheles mosquito. When the infected mosquitoes bite human, the life cycle of plasmodium falciparum starts again.

When Plasmodium falciparum reproduces in human red blood cells, it causes clinical symptoms of malaria. And the liver stages and gametocytes have not symptoms. If stop Plasmodium falciparum before the erythrocytic cycle, it cannot cause symptoms in patients. Reactive oxygen species (ROS) may play a role in the pathogenesis of malaria. Extravasated red blood cells may lead to ROS generation, causing coma and damage to local tissues. In humans, some evidence show that ROS play a role in malaria pathogenesis. In patients with falciparum malaria, especially those with severe disease, monocyte generation of ROS increase [3]. Plasmodium falciparum infected red blood cells produce pyrogenic material which triggers the release of tumour necrosis factor (TNF), increase of TNF cause pyrexia [4]. The timing and amounts of TNF that are released, may be important determinants of malaria symptoms and mortality.

The pre-erythrocytic Plasmodium falciparum vaccine RTS,S was reviewed by the European Medicines Agency, marking the first human vaccine against malaria to pass regulatory scrutiny [5]. RTS,S is pre-erythrocytic vaccine (PEV), the target antigens of PEV from Plasmodium sporozoite and liver stages. It can induce body produce antibody that against surface antigens of sporozoites. The antibody clears sporozoites from skin or blood, and block their invasion of liver cells. The phase III trial delivered 3 doses at 1-month according to immunization schedule in 15,459 children from seven African countries, and add a booster dose 18 months after the third dose. After inoculated 4 doses, clinical malaria episodes were reduced by about 36% in young children and about 26% among infants. In the first 6 mouths, the pathogenicity rate of clinical malaria reduces 68% [6].

Despite the progress, efforts to control malaria still face many challenges. The RTS,S vaccine not only has a limited protective effect (30%-50% protection rate), but also a short duration of protective immunity (6-8 months) [6]. The protective effect of RTS,S/ASO1 vaccine was inversely correlated with malaria epidemic intensity and the host's previous malaria exposure. Therefore, the actual protective effect of the vaccine in large-scale use in areas with high malaria prevalence is likely to be weaker than the results of phase III clinical trials. When it was used in large populations, the plasmodium antigens may mutate, which reducing the protective effectiveness of the RTS,S vaccine. The development of new malaria vaccines continues apace. Some new vaccines seek to improve the protective efficacy of RTS, S, and compensate for the short duration of effective immunity. Based on the life cycle of the malaria parasite, except PEV, which act on exo-erythrocytic stage, there are 2 other pathways to design vaccine. It can stop parasites in mosquito stage and in erythrocytic stage. According to the pathogenesis of Plasmodium falciparum, the parasite cause symptoms in human body in erythrocytic stage. The method of gene editing makes the malaria parasite unable to invade red blood cells in the human body, but still retains the antigenic properties of the malaria parasite. This is a feasible approach to vaccine design.

This research proposes a whole merozoite vaccine as a solution to the limitations of current malaria vaccines. The vaccine's primary antigen is a merozoite lacking a specific protein, achieved through CRISPR-Cas9 technology that knocks out the corresponding protein fragment. This deficiency prevents the merozoite from invading red blood cells, causing it to remain on the cell surface without progressing to the next stage of replication, thereby greatly reducing its pathogenicity. As a result, the antigen is fully exposed to the immune system. Because the transgenic merozoite closely mimics

the natural merozoite, it effectively stimulates the production of potent antibodies against the blood stage of malaria. The vaccine's excipients are fairly conventional, with physiological saline as the primary excipient, aluminum salts as the adjuvant, and 2-phenoxyethanol as the preservative. If successful, this vaccine could offer a highly effective, durable, and cost-efficient method for malaria prevention.

2. Malaria vaccine design

Considering that some regions in Africa are economically impoverished and unable to afford existing malaria treatment methods, we avoided mainstream approaches. Techniques such as monoclonal antibodies and antigen structure-targeting technologies are not utilized. Our novel malaria vaccine design is inspired by traditional attenuated vaccines. Single-dose administration of attenuated vaccines provides long-term immunity, making them very suitable for areas with a high incidence of malaria. The vaccine targets the malaria blood stage when the disease occurs. Merozoites are the antigens during the blood stage, so the main ingredient of our vaccine should be attenuated merozoites to induce an immune response closely resembling that of a natural infection. Merozoite surface antigens exhibit high variability; thus, using whole merozoites as vaccine antigens should be more effective. Malaria vaccines based on the principle of attenuated vaccines have already made significant progress. The most well-known whole sporozoite vaccine (WSV) is the PfSPZ series, which has a long history, and some of its products have now advanced to the clinical stage [7]. They modify the structure of sporozoites using radiation and gene knockout techniques to create attenuated antigens. Here, we aim to offer a new perspective.

The design is to prevent merozoites from entering red blood cells, thereby blocking the pathogen's replication process and preventing the onset of disease. Merozoites that fail to successfully enter red blood cells will adhere to the exterior of the red cells, becoming exposed to the immune system. This exposure allows the immune system to recognize and mount an immune response against them. Scientists have discovered that merozoite invasion is associated with certain molecular events [8]. In simple terms, merozoites first contact red blood cells, causing slight deformation. This is followed by the opening of calcium ion channels and the observation of calcium influx, after which the merozoites enter the red blood cells. Merozoite surface proteins have been shown to be highly associated with certain molecular events. If certain specific proteins can be disrupted, thereby preventing these molecular events, the entry of merozoites into red blood cells can be halted. Merozoite surface proteins are numerous, and the functions of a significant portion of them are not well understood [9]. Therefore, we have conducted a screening from the limited number of proteins with clearly defined functions.

MSP1 is highly associated with red blood cell invasion and is the most abundant among GPI-anchored proteins. It binds extensively to various proteins at different stages [10], forming different protein complexes and participating in numerous merozoite activities. Therefore, it cannot be chosen as a target for disruption, as it may affect the vaccine antibody’s activity and overall structure. One rhoptry protein, PfRH5, and two microneme proteins, PfRipr and CyRPA, bind together to form a complex [11]. This complex can trigger changes in calcium influx between red blood cells and merozoites. This means that disrupting any one of these three proteins can block the calcium influx changes, preventing merozoites from entering red blood cells. The CyRPA protein was chosen because it is highly conserved and lacks immune pressure. The disrupted CyRPA protein produces antigens that are less likely to mutate or refold, offering good stability. The CyRPA protein is present in small amounts among surface proteins, so disrupting it has minimal impact on the overall structure and function of the antigen, potentially making the vaccine more effective.

Regarding how to disrupt CYRPA, genetic engineering would be the most efficient and economical method. Traditional chemical methods, which involve soaking the entire antigen in chemical reagents, would disrupt the entire antigen's structural activity and cannot precisely modify the target site. Precision chemical modifications, such as using nanomaterials to target specific sites, are not yet

mature and, even if successful, are costly. Genetic engineering is preferred. Transgenic antigens can be proliferated in environments that simulate in vivo conditions, making purification easier, costs lower, and quality more stable. Using CRISPR-Cas9 technology, guide RNA is used to target the region near the CyRPA protein expression gene. The Cas9 protein is then used to knock out the CyRPA gene. By doing this, antigens with abnormal CyRPA protein function expression can be obtained.

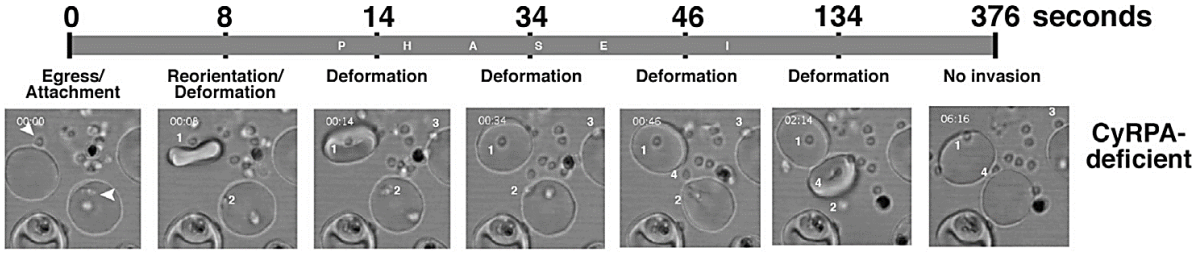

Figure 1. Delay images of CyRPA defective merozoites attempting to invade [8]

In order to explore the function of the complex formed by RfRH5, CYRPA, and PfRipr, it can individually knock out the protein expression genes. Merozoites could not enter red blood cells when the CyRPA gene was knocked out (Figure 1 and 2) [8]. This result provides feasibility for our approach. Whether to use transgenic sporozoites or transgenic merozoites as the main component of our vaccine was considered. If transgenic sporozoites are used, the first issue our vaccine might encounter is the same as previous whole sporozoite vaccines, where the injection method might require mosquito bites, which is impractical. However, its advantage is that after undergoing one replication in the liver stage, the number of merozoites produced will greatly increase, making our vaccine more effective, requiring fewer doses, and better simulating the natural infection route. If transgenic merozoites are used as the main component of the vaccine, the injection method can be intravenous. The concern, however, is whether the injected transgenic merozoites will distribute evenly throughout the human body, which still needs experimental validation.

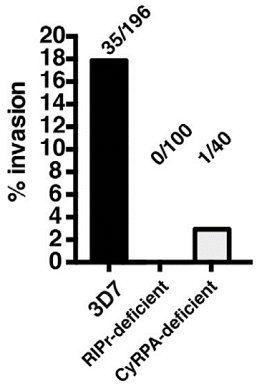

Figure 2. Delay images of CyRPA defective merozoites attempting to invade [8]

There are also differences in the production and cultivation of sporozoites and merozoites. Cultivating sporozoites is more complex as it requires mosquito propagation, whereas merozoites can be cultured by simulating the blood-stage red blood cell cycle in the human body, allowing to produce multiple generations. Given that the administration of a whole sporozoite vaccine via mosquito bites is complex and unreliable, and also faces numerous production challenges along with high costs for subsequent storage, it will focus on the concept of a whole merozoite vaccine (WMP) instead. The primary components of the vaccine include the transgenic deficient CYRPA merozoite as the antigen, 2-phenoxyethanol as the preservative, aluminum salts as the adjuvant, and physiological saline as the excipient. Compared to the whole sporozoite vaccine, this formulation offers several advantages. It has a simpler composition, is easier to store, and can be administered via intravenous injection.

3. Security and advantages analysis

To test the safety and effectiveness of a malaria vaccine, both mice and monkeys are used as animal models. To monitor potential side effects, the vital signs and behaviors of the animals will be closely observed throughout the experiment. This will include recording physiological indicators such as heart rate, respiration rate, and body temperature. Additionally, the concentration of antibodies will be measured after the vaccine has been injected, which can ensure that the mice and monkeys generate sufficient levels of antibodies against Plasmodium. This approach will provide critical data on the vaccine's immunogenicity and potential adverse effects, contributing to a comprehensive understanding of its safety and efficacy.

According to the FDA standard of vaccine approval, three stages of volunteer study are necessary [12]. In our case, the first stage includes injecting our vaccine into 20-50 volunteers and determine the average maximum tolerated dose (MTD) of people. The adverse effects should be monitored when reaching the MTD. With the use of genetically modified Plasmodium merozoites, real-time imaging makes it possible for us to monitor the development of Plasmodium parasites in the liver stage in human beings in the stage 1. The second stage of our study aims to test our vaccine effectiveness and safety on hundreds of volunteers from all over the world. Standardized test is also projected to evaluate the immune response of our vaccine. The third stage of our study contains the study and test on thousands of volunteers to show whether our vaccine decreases the incidence rate of malaria [13].

There are several concerns to the development stage of our vaccine. The first possible challenge is the red blood cell deformation. The red blood cell deformation happens after the Plasmodium merozoites attaching to the red blood cells [14]. Although the protein deficiency decreases the Plasmodium merozoites ability of red blood cell deformation, 60% of the CyRPA-deficient merozoites will deform the red blood cells after attachment [15]. The red blood cell deformation results in some serious adverse effects. Deformed red blood cells may adhere to the blood vessel wall, causing thrombosis and obstructing blood circulation. Excessive deformation can damage the red blood cell membrane, leading to hemolysis and the release of hemoglobin, which may cause jaundice [16]. Such safety concerns will be the major research object of our further study.

Even though the mainly functional proteins are deficient, some merozoites can still enter the red blood cells. The 2.5% of the CyRPA-deficient merozoites are able to invade into the red blood cells [15]. Such process has two sides. The invasion can let the merozoites enter the blood stage and reproduce, which can stimulate the immune response [17]. If the merozoites number is out of control due to the invasion, it is particularly dangerous because malaria-like symptoms such as fever and fatigue will occur on the patients [18]. The merozoites reproduction may cause the red blood cell rupture, which leads to anemia and other complications [16].

Considering such flaws of our design. Further study can target on another protein PfRipr, which combines with other protein and contributes to invade the red blood cells [19]. Antibodies targeting the EGF like domain at the C-terminus of PfRipr protein can inhibit the process of red blood cell invasion, thereby preventing infection by malaria parasites [19]. Inhibiting PfRipr has an advantage that there will be no risk of red blood cell invasion.5 However, 93% of the merozoites will cause the red blood cell to deform, which means that the risk of thrombosis and hemolysis becomes higher [15].

One of the most advanced and marketed malaria vaccine candidates is RTS,S/AS01, which is a vaccine targeting the surface protein RTS,S of the malaria parasite, combined with the AS01 adjuvant. However, the RTS,S/AS01 vaccine isn't fully superior and has several drawbacks. For limited protection, in clinical trials, the vaccine provided 39.3% protection for infants aged 6-12 months and 28.3% protection for children aged 5-17 months, which is a low protection [20]. For the protection decreases over time, after vaccination, the protection gradually decreases over time. At 7 months after vaccination, the protection had dropped to 26.7% [21]. For safety issues, the vaccine has had some serious adverse reactions, such as meningitis, and its long-term safety needs to be further evaluated.

In our design, we devote ourselves to solve the problems. The processed merozoites are expected to be an excellent type of antigen which help the body to stimulate immune system and provide sufficient protection against potential disease. In the red blood cells, the reproduced merozoites are expected to give durable protection to human beings. Although the processed merozoites may lead to malaria-like symptoms, most of them are considered to be mild and non-lethal.

4. Conclusion

For Plasmodium falciparum, which still causes large numbers of cases and deaths in sub-Saharan Africa. Although the existing vaccine RTS,S on the market has been successful in reducing the clinical risk of malaria in African children, it still has shortcomings, such as limited protective effect and short duration of protective immunity. Based on studies of specific proteins on the surface of the malaria parasite, three specific functional proteins, PfRH5, PfRipr and CyRPA, are associated with Plasmodium invasion of red blood cells. Breaking one of these proteins prevents the parasite from invading red blood cells. This provides an idea for designing new vaccines. CyRPA protein is highly conserved and lacks immune stress, and plasmodium that destroy this protein are more stable and retain antigenic properties. This new type vaccine is expected to provide more adequate and longer immunity protection, and will not cause serious side effects.

Authors Contribution

All the authors contributed equally and their names were listed in alphabetical order.

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