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Immunogenicity and *in vitro* protective efficacy of a polyepitope *Plasmodium falciparum* candidate vaccine constructed by epitope shuffling

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Abstract

A polyepitope chimeric antigen incorporating multiple protective and conservative epitopes from multiple antigens of *Plasmodium falciparum* has been considered to be more effective in inducing multiple layers of immunity against malaria than a single stage- or single antigen-based vaccine. By modifying the molecular breeding approach to epitope shuffling, we have constructed a polyepitope chimeric gene that encodes 11 B-cell and T-cell proliferative epitope peptides derived from eight key antigens mostly in the blood stage of *Plasmodium falciparum*. A 35-kDa antigen encoded by this gene, named Malaria RCAg-1, was purified from an *E. coli* expression system. Immunization of rabbits and mice with the purified protein in the presence of Freund's adjuvant strongly generated long-lasting antibody responses that recognized the corresponding individual epitope peptide in this vaccine as well as blood stage parasites. $CD4^+$ T-cell responses were also elicited as shown by the enhancement of T-cell proliferation, IFN-γ and IL-4 level. *In vitro* assay of protection revealed that vaccine-elicited antibodies could efficiently inhibit the growth of blood-stage parasites. Additionally, the chimeric antigen was recognized by human serum specimens from malaria patients and individuals living in the endemic area. Our studies indicate the potential of M.RCAg-1 recombinant protein as malaria candidate vaccines as well as the rationale of the epitope shuffling technology applied in designing malaria vaccines.

Keywords: Malaria vaccine; DNA shuffling; Immunogenicity; Protective efficacy

1. Introduction

Malaria, one of the most serious infectious diseases worldwide, has been a threat to humans for millennia. In tropical and sub-tropical areas an estimated 500 million people are infected yearly with up to three million deaths [1]. *Plasmodium falciparum*, one of the four human malaria species, produces the highest morbidity and mortality, with 80% of cases being in pregnant women and children under 5 years of age. Widespread development of drug-resistant forms of parasites, development of insecticide-resistant *Anopheles* mosquito vectors, increasing movement of population between the endemic and non-endemic areas, and global warming continue to worsen the situation. There is a pressing need for efficacious strategies to control and prevent malaria, including the development of a malaria vaccine as one of the most sustainable tools to protect more than 40% of the world population.

The feasibility of developing a malaria vaccine is based on observations that immunization with irradiated

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sporozoites protects volunteers and animal models against infected mosquito challenge; that humans exposed to repeated infections in the malaria-endemic areas gradually develop natural protection; and that experiments have demonstrated the effectiveness of passive transfer of immunity. At least 40 promising protective antigens from malaria parasites have been identified, and three types of malaria vaccines targeting different stages of the parasite life cycle are under development: pre-erythrocyte, asexual and sexual blood stages. Although some have shown promise, the success is still far off. The complications include the genetic complexity of the parasites; the subsets of parasite antigens which the immune system must combat at each stage; the strategies that the parasites have evolved to evade the host immune system; and the simultaneous occurrence of multiple malaria infections, not only of different species but also of different strains. For these reasons, we believe that an ideal malaria vaccine must combine antigens from either all three stages of the malaria parasite's life cycle, or epitopes of as many key antigens as possible, to induce both the humoral and the cellular immunity required for optimal efficacy.

So far, several multi-component malaria vaccines have been tested in clinical trials in humans since the 1980s: (1) Spf66 [2], a chemically synthesized *P. falciparum* vaccine containing different portions of several blood stage proteins, and linked by repeat sequence from the pre-erythrocyte CS protein. Protection conferred by this vaccine was not proved in a phase I/IIa trial; (2) NYVAC-pf7 [3], a multistage *P. falciparum* vaccine containing seven stage-specific antigens and expressed in vaccine–virus system. Poor antibody responses were elicited in the volunteers and the immune protection was unsatisfactory; (3) a polyepitope vaccine incorporating 15 cytotoxic T lymphocyte epitopes. It was able to prime protective CTL responses in mice administrated one dose without adjuvant [4]; (4) FALVAC-1, a multivalent vaccine incorporating 21 different B-cell, T-cell, and CTL epitopes from seven different antigens of *P. falciparum* expressed in three stages of the parasite life cycle. This vaccine in rabbits showed promising anti-parasitic activity with monocytes *in vitro*. However, only FALVAC-1/Freund's adjuvants reduced the parasitemia and extended the number of days to treatment while none of other antigen–adjuvant combinations could provide discernable levels of protection in *Aotus nancymai* monkeys [5–7]. Other multicomponent vaccines are still in the process of clinic trials and awaiting confirmation of their efficacy which include the multi-stage pre-erythrocytic DNA vaccine (Vical/NMRC), recombinant FMP-1 plus RTS, S MSP-1 3D7+CSP(WRAIR), MVA prime-boost DNA Multi-epitope string +TRAP (Oxford) and MVA prime-boost FowI Pox 9 Multi-epitope string +TRAP (Oxford) [8].

In general, these multicomponent vaccines belong to three main types: (1) "string-bean" polypeptide containing CTL epitopes and the CTL responses elicited were sufficient; (2) polypeptide incorporating multiple epitopes for both humoral and cellular immune response, in which the antibody level elicited by the antigen itself was not strong enough without a suitable adjuvant; and (3) polypeptide, which needs to be fused with a carrier protein for optimizing its immune efficacy. However, aside from vaccination with whole genomic expression libraries [9], so far few individual chimeric antigens incorporating B-cell and T-cell epitopes without a fusion protein have shown strong efficiency in eliciting a B-cell response, which is the most important immune protection against blood stage malaria parasites [10]. Therefore, an effective blood stage malaria vaccine has to mimic the natural protective immunity that develops in people living in malaria-endemic areas, and evoke the immune system to impact the merozoite as it continues in its replicative cycle. Although the host may still get malaria and become sick, the severity and lethality of the disease would be diminished. Ultimately, in this stage of infection, it is well known that not only antibodies directed against the parasite are essential, but also cell-mediated mechanisms are critical for the proper acquisition of acquired immunity against malaria [11].

Our previous experiments have indicated that the addition or removal of a single epitope from a short polypeptide containing a few epitopes, could affect the antibody level as well as the T-cell proliferation responses induced by the chimeric polypeptide. This indicates that it is not the number of epitopes but the configuration of the molecule that plays an essential role in the efficiency of a chimeric antigen [12]. Other groups have also reported that a stable molecular conformation is required for some candidate vaccines to provide protection against parasite challenge (such as the disulfide bond formation in 3D structure) [13]. Unfortunately, it is not easy to control the subtle substructure of polyepitope proteins when the epitopes are linked in tandem. Here, we have constructed chimeric antigen molecules using the technique of molecular breeding of 14 shuffled epitopes, most of which are derived from vaccine candidate antigens of the blood stage of *P. falciparum* and two T helper epitopes being selected from antigens of sporozoits just in order to provide help for antibody production. Previous studies in our lab have shown that *in vivo* DNA library vaccination of polyeptiope chimeric genes with 1.2 and 2.0 kb sizes have the best immunogenicity in rodent model [14]. One of chimeric antigen genes, named M.RCAg-1 was identified by screening for antigenicity and immunogenicity from these two DNA libraries both *in vitro* and *in vivo*.

In the present study, we examined the immunological and vaccinological properties of the corresponding *E. coli*-expressed recombinant polyepitope protein, M.RCAg-1, as a candidate vaccine by four aspects below: (i) antibody and T-cell responses in mice and rabbits; (ii) immunoreactivities of the vaccine-elicited antibodies against the blood stage of *P. falciparum*; (iii) the reactivity of the chimeric antigen with sera of malaria patients or residents living in highly endemic area; and (iv) *in vitro* antiparasite activity of vaccine-elicited antibodies.