Molecular Immunology 90 (2017) 158–171

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Biological, immunological and functional properties of two novel multi-variant chimeric recombinant proteins of CSP antigens for vaccine development against *Plasmodium vivax* infection

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ARTICLE INFO

Keywords:
Plasmodium vivax
Vaccine
Circumsporozoite protein
Chimeric VK210/VK247
Conformational structure
Biological function

ABSTRACT

The circumsporozoite protein (CSP) of the malaria parasite *Plasmodium vivax* is a major pre-erythrocyte vaccine candidate. The protein has a central repeat region that belongs to one of repeat families (VK210, VK247, and the *P. vivax*-like). In the present study, computer modelling was employed to select chimeric proteins, comprising the conserved regions and different arrangements of the repeat elements (VK210 and VK247), whose structure is similar to that of the native counterparts. DNA encoding the selected chimeras (named CS127 and CS712) were synthetically constructed based on *E. coli* codons, then cloned and expressed. Mouse monoclonal antibodies (mAbs; anti-Pv-210-CDC and −Pv-247-CDC), recognized the chimeric antigens in ELISA, indicating correct conformation and accessibility of the B-cell epitopes. ELISA using IgG from plasma samples collected from 221 Iranian patients with acute *P. vivax* showed that only 49.32% of the samples reacted to both CS127 and CS712 proteins. The dominant subclass for the two chimeras was IgG1 (48% of the positive responders, OD_{492} = 0.777 ± 0.420 for CS127; 48.41% of the positive responders, OD_{492} = 0.862 ± 0.423 for CS712, with no statistically significant difference $P > 0.05$; Wilcoxon signed ranks test). Binding assays showed that both chimeric proteins bound to immobilized heparan sulphate and HepG2 hepatocyte cells in a concentration-dependent manner, saturable at 80 µg/mL. Additionally, anti-CS127 and −CS712 antibodies raised in mice recognized the native protein on the surface of *P. vivax* sporozoite with high intensity, confirming the presence of common epitopes between the recombinant forms and the native proteins. In summary, despite structural differences at the molecular level, the expression levels of both chimeras were satisfactory, and their conformational structure retained biological function, thus supporting their potential for use in the development of vivax-based vaccine.

1. Introduction

Plasmodium vivax is the most widespread species of human malaria outside Africa and is predominant in countries where malaria is in the pre-elimination or elimination phases (WHO, 2014). *P. vivax* parasites are capable of causing delayed infections through the formation of dormant the liver stages, known as hypnozoites, that can activate several months after the initial infective bite, causing further disease and increasing the potential for transmission (Robert et al., 2005). This poses a major obstacle to control and elimination programs. It is now recognized that *P. vivax* infection can lead to severe clinical symptoms and mortality (Lomar et al., 2005; Rodriguez-Morales et al., 2006; Tjitra et al., 2008; Price et al., 2009; Kochar et al., 2009; Nadkar et al., 2012). Therefore, there is a need for more efforts and investments to develop a vaccine to help control, and eventually eliminate *P. vivax*.

One of the approaches for a malaria vaccine is to elicit sterile

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http://dx.doi.org/10.1016/j.molimm.2017.06.033
Received 1 March 2017; Received in revised form 31 May 2017; Accepted 19 June 2017

immunity against the pre-erythrocytic stage (Heppner, 2013) by blocking invasion of hepatocytes by sporozoite and/or the development of the hepatic stages thereby preventing the blood infection (Hollingdale, 1990; Heppner, 2013). Previous experimental vaccinations with irradiation-attenuated sporozoite induced sterile protection against a sporozoite challenge in animal models and in humans (Clyde, 1975; Hoffman et al., 2002). Recently, methods have been developed to produce sufficient quantities of *P. falciparum* sporozoites for large-scale vaccination with irradiation-attenuated sporozoites (Luke and Hoffman, 2003). However, as *P. vivax* cannot be maintained in *in vitro*, a sporozoite-based vaccine is not a practical option, thus current efforts have focused on developing a vivax-based subunit vaccine.

The most abundant protein on the surface of all *Plasmodium* sporozoites is the circumsporozoite protein (CSP). It is involved in the motility from the site of inoculation to the invasion of the hepatocyte (Nussenzweig and Nussenzweig, 1985; Pancake et al., 1992; Mota and Rodriguez, 2004; Kappe et al., 2004; Coppi et al., 2011). This protein is the main candidate of pre-erythrocyte recombinant malaria vaccine (Qari et al., 1993; Gonzalez et al., 2001; Herrera et al., 2007; WHO, 2012), and antibodies against it have been shown to mediate protection against *Plasmodium* sporozoite challenge in preclinical models and humans (Charoenvit et al., 1991; Potocnjak et al., 1980; Tam et al., 1990; Zavala et al., 1987; Kebaier et al., 2009; Kester et al., 2009). Additionally, much evidence has indicated that immune responses targeting the *P. vivax* CSP (PvCSP) are likely play a crucial role in mediating protection against *P. vivax* in clinical and preclinical studies (McCarthy and Clyde, 1977; Charoenvit et al., 1991; Yang et al., 1997).

The PvCSP comprises two conserved domains that flank a central repeat region that belongs to one of three different families (VK210, VK247, and the *P. vivax*-like variants) (Rosenberg et al., 1989; Qari et al., 1993; Gopinath et al., 1994). The VK210 and VK247 families are the most predominant, and it was previously shown that they are not immunologically cross-reactive (Rosenberg et al., 1989), and can thus contribute to the development of strain-specific immunity (Machado et al., 2003). Therefore, a multi-variant chimeric subunit vaccine would be required. However, such a subunit vaccine might have limited intrinsic immunostimulatory properties that could result from the structural modifications inherent to its design, which would then diminished the ability of the elicited antibodies to recognize all variants of the native target antigen. Thus, it is important to point out that for development of a PvCSP-based vaccine; a well-established strategy is needed to enhance the immunogenicity of neutralizing B-cell epitopes.

In the late 1980s and the early 1990s, immunization with a recombinant PvCSP expressed in yeast induced inadequate immunity in non-human primates (Barr et al., 1987) and in humans (Collins et al., 1989; Herrington et al., 1991). Later, multiple antigen constructs were used to develop epitope-based vaccines using the vivax repeat motif, and some levels of protection were observed in Saimiri monkeys (Yang et al., 1997; Collins et al., 1997). Subsequently, synthetic peptide-based vaccines were considered and tested in non-human primates and humans (Udhayakumar et al., 1998; Herrera et al., 1997, 2005) to improve the immunogenicity of the PvCSP-based vaccine and to overcome production difficulties. Recently, a study by Yadava et al. (2007) reported a new PvCSP chimera (named VMP001) that was produced in *Escherichia coli* (*E. coli*), and immunization of mice with this chimera formulated with Montanide ISA-720 showed it to be immunogenic and to recognize live sporozoites (Yadava et al., 2007; Bell et al., 2009). However, *in vitro* evaluation of antibodies to VK247 and VK210 peptides showed that the response to the VK247 was lower than that to the VK210 peptide, maybe because of a lower VK247 copy numbers of the repeats in this vaccine construct (Vanloubbeeck et al., 2013). Furthermore, recent humans phase 1 clinical trial using VMP001/AS01B failed to induce sterile protection, and a significant delay in the time to detectable parasitaemia was observed in only 59% of vaccinated subjects (Bennett et al., 2016).

In order to obtain a recombinant chimeric antigen comparable in its immunogenicity to its native form, we designed and constructed two novel synthetic chimeric genes of *P. vivax* CSP based on the two most widespread variant forms (VK210 and VK247). Both synthetic structures consisted of the native protein's conserved N-terminal and C-terminal regions, flanking a truncated repeat region of the either VK210/VK247 (named CS127) or VK247/VK210 (named CS712). In order to select the most appropriate chimeric protein structure similar to their native proteins in which both have biological function, computer modelling was employed to guide the design of CS127 and CS712 towards physicochemical stability, including RNA stability, epitope mapping, prediction of tertiary structure, and comparison of structure of the both chimeric proteins. The predicted 3-dimensional (3D) structure was also compared to that of natural proteins of similar size to ensure that the conformation of the two synthetic proteins would be similar to their natural counterparts in terms of internal energy. These modelling studies led to the selection of the amino acid sequences of the two constructs. The nucleotide sequences for both the synthetic *Pvcs127* and *Pvcs712* genes were based on those of the *E. coli* codons in order to optimize expression levels. Subsequent to the expression and purification of the two chimeric recombinant proteins, their potential usefulness for the development of a protective vivax malaria vaccine, was assessed by evaluation of their biological and functional properties using enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence antibody test (IFAT), as well as heparan sulphate and hepatocyte-binding assays.

2. Materials and methods

2.1. Designing chimeric CSPVK210/VK247 proteins

In this investigation, two synthetic *Pvcsp* genes were designed based on the complete gene and protein sequences of VK210 (Sal-1, accession no. GU339059) and VK247 (Papua New Guinea [PNG], accession no. M69059) sequences (Fig. 1A and 1B). The first construct (named after CS127 based on the number and the tandem of motif repeats; accession no. KY548403) consisted of 76 conserved N-terminal amino acids (aa: 20–95, Sal-1 sequence, accession no. GU339059), followed by EDGAGNQPG sequence of VK247 (aa: 96–104; PNG, accession no. M69059) and the first 12 repeats of VK210 (GDRA[D/A]GQPA), followed by the 7 repeats of VK247 (ANGAG[N/D]QPG) (Fig. 1C). Moreover, the post-repeat region of this newly construct was considered to have one copy of 12 inserted amino acid sequences, GGNAANKKAEDA, which is detected in South Korean and Iranian isolates (aa: 267–292, Belem sequence, accession no. EU401923) and conserved C-terminus (aa: 281–350; Sal-1 sequence, accession no.GU339059) (Fig. 1C). For the second construct, (named after CS712 based on the number and the tandem of motif repeats; accession no. KY548404), both N- and C-terminals were the same as the first designed construct. However, the arrangement of the repeats in CS712 was four of ANGAGNQPG and three of ANGAGDQPG repeats of VK247 (totally, 7 repeat types of VK247), followed by six of each VK210 (GDRA[A/D]GQPA) (totally, 12 repeat types of VK210) (Fig. 1D).

To design both constructs, Sal-1 and PNG reference sequences were retrieved from the GenBank and aligned with ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Thompson et al., 1994) to define conserved and non-conserved regions. Then the both genes were synthesized based on *E. coli* codon preferences (Sharp and Li, 1987). Two restriction endonuclease recognition sites (*Nde*I for 5′ end and *Hind*III for 3′ end) were inserted to synthetic constructs as well as hexa-histidine (6 × His)-tag at the C-terminal site to facilitate recombinant protein purification by nickel affinity protein purification. Both constructs were synthesized and delivered as two separate clones in pET26b plasmid by ShineGene Molecular Biotech, Inc. (Shanghai, China). The secondary structure of messenger RNA of the chimeric genes was analyzed by Mfold (version 2.3 energies) online software at http://www.bioinfo.rpi.edu/applications/mfold (Zuker, 2003).