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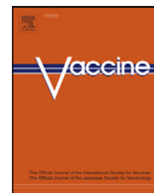

High immunogenecity and erythrocyte-binding activity in the tryptophan-rich domain (TRD) of the 74-kDa *Plasmodium vivax* alanine-tryptophan-rich antigen (PvATRAg74)

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ABSTRACT

Plasmodium vivax is the most widespread species of human malaria parasite affecting 70–80 million people worldwide each year. In recent years, some potential vaccine candidate antigens from *P. vivax* have been identified including tryptophan-rich antigens PvTRAg and PvTARAg55. We report here the identification and partial characterization of a 74 kDa *P. vivax* alanine-tryptophan-rich antigen (PvATRAg74) which is expressed by all asexual blood stages of the parasite. This protein contains two major domains, i.e. alanine-rich domain (ARD) in N-terminal region and the tryptophan-rich domain (TRD) at C-terminus. PvATRAg74 also contains variable numbers of octa-peptide repeats in the ARD region. The C-terminal PvATRAg74 containing TRD was highly conserved among 32 *P. vivax* isolates while N-terminal ARD showed genetic polymorphisms. The 36 kDa TRD was expressed in *E. coli* and named here as His_6-TRD. The purified recombinant His_6-TRD showed binding with uninfected human erythrocytes. This antigen was also recognized by all 38 *P. vivax* patients' sera on ELISA thus showing a very high seropositivity rates. In vitro stimulation of lymphocytes with purified His_6-TRD indicated that it induced T cell immune response in majority (94%, $n = 16$) of *P. vivax* exposed individuals. The stimulated T cells produced higher amount of IL-4 and IL-10 than IFN-γ, TNF-α, and IL-12 indicating a Th2 type of response bias. Unlike PvTARAg55, this antigen is more immunogenic in humans and possesses the erythrocyte-binding activity. Immunogenecity of PvATRAg74 is similar to PvTRAg whose erythrocyte-binding activity still remains unknown.

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1. Introduction

Plasmodium vivax is the most widespread species of the human malaria parasite affecting 70–80 million people each year in the tropical countries [1]. Progress on vaccine development for this parasite has been slow partly due to its non-cultivable nature. Only limited numbers of vaccine antigens are characterized from *P. vivax* therefore, it is desirable to identify and characterize other potential molecules from this parasite. Tryptophan-rich proteins of various *Plasmodium* species have been suggested to be the potential vaccine candidates [2–5]. The current genomic sequence database of *P. vivax* contain more than 20 putative tryptophan-rich proteins (http://www.plasmodb.org/) which need to be characterized for their vaccine potential.

The tryptophan-rich antigens were first reported from *P. yoelii* [*P. yoelii* particulate antigen (PypAg-1 and PypAg-3)] [5–7] and then its orthologues from *P. falciparum* [tryptophan-threonine antigen (TryThrA); merozoite-associated tryptophan-rich antigen (MaTrA); tryptophan-rich antigen-3 (TrpA-3) and lysine-tryptophan-rich antigen (LysTrpA)] [4,8,9] and *P. vivax* [*P. vivax* tryptophan-rich antigen (PvTRAg), *P. vivax* tryptophan-alanine-rich antigen (PvTARAg55)] [2,3]. The PypAg-1 and PypAg-3 are expressed in the cytoplasm and associated with the membrane of *P. yoelii* infected erythrocytes [5,6]. They also bind to the uninfected erythrocytes suggesting their possible role in the parasite re-invasion process [5,6]. Most importantly, *in vivo* studies in mice have shown them to confer protective immunity against *P. yoelii* infection [5,6]. The PfTryThrA is expressed almost throughout the entire blood stages of the parasite and considered to be an orthologue of PypAg-1 (∼33% sequence identity) whereas MaTrA (35 kDa) show high sequence identity (∼25%, limited to tryptophan-rich region) with PypAg-3 [4–6,8]. Unlike TryThrA, MaTrA is expressed in the late schizonts and merozoite stages of the parasite [8]. Further study on TryThrA led to the identification of four high activity binding peptides (HABPs) which show specific binding with normal human erythrocytes [10]. These HABPs also inhibit in vitro merozoite invasion,

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suggesting that TryThrA may be involved in merozoite–erythrocyte interaction during invasion [10].

We describe here an alanine-tryptophan-rich antigen (PvATRAg74) from *P. vivax* which contains a variable alanine-rich domain (ARD) at N-terminus and a highly conserved tryptophan-rich domain (TRD) at C-terminus. Recombinant tryptophan-rich domain (TRD) of the PvATRAg74 contains erythrocyte-binding activity and also reacts strongly with sera from *P. vivax* infected patients as well as induces high cellular immune response among the *P. vivax* exposed individuals. The PvATRAg74 shows a higher immune response than the recently described PvTARAg55, however, these two antigens share very little homology [2].

2. Material and methods

2.1. *P. vivax genomic database mining and identification of the ORF encoding PvATRAg74*

The BLASTP (Basic Local Alignment Search Tool using a Protein query) search using PvTRAg [3] amino acid sequence was performed at *P. vivax* genomic sequence database (http://www.plasmodb.org). One of several putative tryptophan-rich protein-encoding ORFs present in the database is 1938 base pairs ORF which encodes for a 74-kDa protein. The ORF was also analyzed for the presence of predicted N-terminal signal sequence on SignalP 3.0 server [11,12], trans-membrane helices [13], motifs and domains (http://www.us.expasy.ch) and tandem repeats [14]. BLASTP search was also done at PlasmoDB in order to find out the PvATRAg74's homologues in other *Plasmodium* species.

2.2. *Cloning and expression of tryptophan-rich domain (TRD) of the PvATRAg74*

The 880 bp region encoding the C-terminal tryptophan-rich domain (TRD) of the PvATRAg74 was PCR amplified from *P. vivax* genomic DNA using SpeIF (5′-AGAACTAGTGAATATGACACAG-3′) and Hind75R (5′-TGATTGGATCGAAGCTTAATATG-3′) primers with SpeI and HindIII restriction sites, respectively. The conditions for PCR cycling were: initial DNA denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 51 °C for 1 min and extension at 68 °C for 1 min. Final extension was carried out at 68 °C for 10 min. It was cloned into an *E. coli* expression vector pPROEX™ HTa. Expression was achieved in *E. coli* strain BL21 codon plus (DE3)-RP (Stratagene Inc., La Jolla, USA) by growing at 37 °C for 5 h in presence of 1 mM IPTG. The recombinant His_6-TRD protein expressed in soluble fraction was purified using Ni^{2+}-NTA agarose resin according to manufacturer's instructions (Qiagen, GmbH, Germany). The purified protein was quantitated using Bradford's method with BSA as the standard [15]. The identity of the purified His_6-TRD protein was checked on Western blot using anti-His_6 monoclonal antibody.

2.3. *Naturally acquired antibody response to His_6-TRD*

Sera samples collected from 38 *P. vivax* infected individuals were tested for the presence of TRD-specific naturally acquired antibodies by Western blotting as well as ELISA. Sera samples from 30 malaria naïve individuals were also taken as controls. The protocols for Western blotting and ELISA were same as described earlier [2]. The threshold of positivity in ELISA was calculated based on the mean plus 2S.D. of the reactivity of sera from 30 malaria naïve individuals who had no previous history of malaria.

2.4. *Cellular immune response to recombinant His_6-TRD*

2.4.1. *Study sites and human subjects*

Five milliliters of venous blood were collected from 16 confirmed *P. vivax* exposed individuals (9 male, 7 female; mean age ± standard deviation, 20.6 ± 9.7 years) who had recovered from their last malaria episodes about 8–10 weeks prior to study. All 16 individuals were living in the malaria endemic region in and around Jabalpur, Madhya Pradesh, India. We also obtained blood samples from eight malaria naïve individuals (five male, three female; mean age ± standard deviation, 26.3 ± 4.3 years). Blood collections were made under medical supervision following institutional ethical guidelines as well as with the full informed consent of the donors. All 24 blood samples were found to be parasite negative when checked by microscopy.

2.4.2. *Lymphocyte proliferation assay and cytokine ELISA*

The peripheral blood mononuclear cells (PBMCs) isolation and lymphocyte proliferation assay were performed as described earlier [2]. Lymphocytes were stimulated with 10 μg/ml of the recombinant His_6-TRD antigen. Phytohemagglutinin (PHA) (5 mg/ml) and RPMI medium were used as positive and negative controls, respectively. All culture was done in triplicates. Stimulation index (SI) was calculated as ratio of counts per minute (CPM) of cells stimulated with antigen and the CPM of cells stimulated with medium-only control. As a control, lymphocytes from malaria naïve individuals were included in each set of experiments. The results were presented as a mean ± S.E.M. of triplicate wells.

The culture supernatants were collected at 96 h of antigen stimulation from each triplicate wells and pooled. Levels of TNF-α, IFN-γ, IL-12, IL-4 and IL-10 cytokines were measured (pg/ml) by sandwich enzyme-linked immunosorbent assay (ELISA) using BD OptEIA™ cytokine ELISA kit according to manufacturer's instructions (BD Biosciences San Diego, USA). Quantitative differences in the level of cytokine production were evaluated by the nonparametric Mann–Whitney *U* test using computer graphic software (GraphPad Prism, Version 4.0, San Diego, CA). *P* values less than 0.05 was considered statistically significant.

2.5. *Erythrocyte-binding assay*

The recombinant His-TRD protein was subjected to erythrocyte-binding assay as described earlier [16]. Briefly, 2 million human erythrocyte and lymphocyte cells were allowed to adsorbed onto a 96-well microtitre plates in PBS (pH 7.4) by incubating at 4 °C overnight. After three washing with PBS it was blocked with 5% BSA for 2 h at 37 °C. Various concentrations (0.5, 1, 2, 4, 8, 16, 25, 32 and 50 μg/ml) of purified His_6-TRD were added (100 μl/well) and kept for incubation for 4 h at 37 °C. Wells were again washed three times with PBS and His_6-TRD bound to erythrocytes was recognized with anti-His_6 monoclonal antibody (1:2000 dilutions). Reaction was developed with *o*-phenyldiamine (OPD) substrate (substrate-buffer tablet sets; Sigma–Aldrich, St. Louis, USA) and OD was recorded at 495 nm. A 23-kDa recombinant His_6-thioredoxin protein from *Desulfovibrio desulfuricans* was used as negative control [17]. All experiments were performed in triplicate wells.

2.6. *Indirect-immunofluorescence assay (IFA)*

Immunofluorescence assay was performed using anti-TRD polyclonal antibodies raised in rabbit to study the in vivo expression of the PvATRAg74 antigen in blood stages of *P. vivax* parasite. The protocol used for this assay was same as described ear-

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