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Production of recombinant PvDBPII, receptor binding domain of *Plasmodium vivax* Duffy binding protein, and evaluation of immunogenicity to identify an adjuvant formulation for vaccine development

Rukmini Bhardwaj [a], Ahmad Rushdi Shakri [a], Dhiraj Hans [a], Pankaj Gupta [a], Carmen Fernandez-Becerra [b], Hernando A. del Portillo [b, c], Gaurav Pandey [d], Chetan E. Chitnis [a, *]

[a] International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India
[b] Barcelona Centre for International Health (CRESIB), Barcelona, Spain
[c] Institució Catalana de Recerca I Estudis Avançats (ICREA), Barcelona, Spain
[d] Malaria Vaccine Development Program (MVDP), New Delhi, India

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ABSTRACT

Plasmodium vivax is dependent on interaction with the Duffy antigen receptor for chemokines (DARC) for invasion of human erythrocytes. The *P. vivax* Duffy binding protein (PvDBP) mediates interaction of *P. vivax* merozoites with DARC. The DARC receptor-binding domain lies in a conserved N-terminal cysteine-rich region of PvDBP referred to as region II (PvDBPII). PvDBPII is an attractive vaccine candidate since antibodies raised against PvDBPII block erythrocyte invasion by *P. vivax*. Here, we describe methods to produce recombinant PvDBPII in its correctly folded conformation. A synthetic gene optimized for expression of PvDBPII in Escherichia *coli* and fed batch fermentation process based on exponential feeding strategy was used to achieve high levels of expression of recombinant PvDBPII. Recombinant PvDBPII was isolated from inclusion bodies, refolded by rapid dilution and purified by ion exchange chromatography. Purified recombinant PvDBPII was characterized for identity, purity and functional activity using standardized release assays. Recombinant PvDBPII formulated with various human compatible adjuvants including glycosylpyranosyl lipid A-stable emulsion (GLA-SE) and alhydrogel was used for immunogenicity studies in small animals to downselect a suitable formulation for clinical development. Sera collected from immunized animals were tested for recognition of PvDBPII and inhibition of PvDBPII-DARC binding. GLA-SE formulations of PvDBPII yielded higher ELISA and binding inhibition titres compared to PvDBPII formulated with alhydrogel. These data support further development of a recombinant vaccine for *P. vivax* based on PvDBPII formulated with GLA-SE.

1. Introduction

Malaria remains a major public health problem in many parts of the tropical world. Although, *Plasmodium falciparum* is the most virulent species, *Plasmodium vivax* has a wider geographical spread. Moreover, there is increasing evidence that *P. vivax* can lead to greater number of severe and complicated cases than formerly reported [1]. The availability of a *P. vivax* vaccine will be a valuable cost-effective tool to provide protection against *P. vivax* malaria in endemic countries.

One of the unique features of *P. vivax* biology includes the dependence of *P. vivax* merozoites on interaction with the human Duffy blood group antigen (also known as Duffy Antigen Receptor for Chemokines or DARC) for invasion of human red cells [2]. This key host-parasite interaction is mediated by the interaction of *P. vivax* Duffy binding protein (PvDBP) with DARC as shown in Fig. 1. The receptor-binding domain of PvDBP has been mapped to an amino-terminal conserved, cysteine-rich region of PvDBP that is

* Corresponding author. Current address: Department of Parasites & Insect Vectors, Institut Pasteur, 25-28 Rue du Doctor Roux, Paris 75015, France.
E-mail address: chetan.chitnis@pasteur.fr (C.E. Chitnis).

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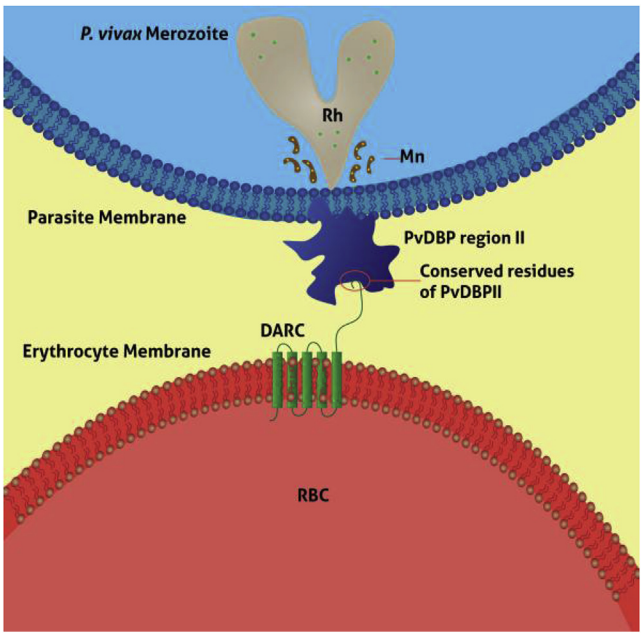

Fig. 1. Interaction of *Plasmodium vivax* Duffy binding protein region II (PvDBPII) with Duffy antigen receptor for chemokines (DARC) on human erythrocytes. RBC-Red blood cell, Rh- Rhoptries, Mn-Micronemes.

referred to as region II (PvDBPII) [3]. PvDBPII-DARC interaction plays a critical role in red cell invasion by *P. vivax* merozoites [2]. Antibodies raised against PvDBPII have been shown to inhibit PvDBPII binding to erythrocytes in a functional assay [4]. In addition, natural acquisition of high titer binding inhibitory antibodies against PvDBPII has been shown to reduce the risk of *P. vivax* infection and reduce *P. vivax* densities in blood in case of infection [5]. These observations support the development of a recombinant vaccine based on PvDBPII.

Here, we describe a process for production of recombinant PvDBPII without any tags for affinity purification. The process includes development of a novel fed-batch fermentation strategy, which instead of using predetermined feed rates to achieve a specific growth rate of 0.12 h^{-1} [6], utilizes a feedback mechanism whereby inputs from measured OD_{600} values during the on-going fermentation are used to control glucose feeding rates to maintain a specific growth rate at 0.12 h^{-1}. This greatly improves the robustness of the feeding strategy. In addition, the process involves isolation and washing of inclusion bodies containing recombinant PvDBPII, refolding of PvDBPII and purification to homogeneity by ion exchange chromatography. Recombinant, purified PvDBPII was characterized for purity, homogeneity and functional activity. Recombinant PvDBPII formulated with adjuvants GLA-SE and alhydrogel were used for immunogenicity studies in small animals to identify a formulation for clinical development.

2. Materials and methods

2.1. Cloning and expression of synthetic gene encoding PvDBPII

A codon optimized synthetic gene encoding PvDBPII region (GenBank Accession No. AAA63423: amino acids from 194 to 521) with C-terminal 6-His tag was previously cloned in pET28(a+) expression vector (Novagen Inc. USA). Primer pair: 5′-GTACTACCATGGATCACAAGAAAACCATC-3′ and 5′-ATCGCTGTCGACTCAGGTAACAACTTCCTGAGTG-3′ was used to amplify DNA encoding PvDBPII by polymerase chain reaction (PCR) using previously described plasmid as template [7]. The amplified product was cloned at NcoI and SalI restriction sites of pET28a(+). The resultant expression plasmid pET28a(+)-PvDBPII was transformed into *E. coli* BLR(DE3) pLysS competent cells (Novagen Inc., USA). Nine transformant colonies were screened for expression of PvDBPII. The colonies were grown in shake-flasks using 50 ml Luria Broth (LB) supplemented with kanamycin (30 μg/ml) at 37 °C. PvDBPII expression was induced by addition of 1 mM IPTG when culture reached an OD_{600} of 0.6–0.8. Cells were harvested four hours post induction. Cell lysates were analyzed by SDS-PAGE and identity of recombinant PvDBPII was confirmed by Western blotting. A clone showing high level expression of recombinant PvDBPII was selected for preparation of research cell banks in completely defined medium and used for fermentation.

2.2. Design of fed batch fermentation process with feedback control

A primary culture of *E. coli* BLR(DE3) pLysS pET28a(+)-PvDBPII grown overnight to an OD_{600} > 3 was used to inoculate two 200 ml secondary seed culture flasks which were grown for 10 h at 37 °C. Both secondary seed flasks at an OD_{600} > 2.5 were used to inoculate a 20 L fermenter (Applikon, The Netherlands) containing 10 L high cell density medium (HCDM) composed of glucose (28 g L^{-1}), $(NH4)_2HPO_4$ (4 g L^{-1}), KH_2PO_4, (13.3 g L^{-1}), citric acid (1.7 g L^{-1}), EDTA (8.4 mg L^{-1}), $MgSO4.7H_2O$ (1.2 g L^{-1}), trace elements (2.5 ml L^{-1}), thiamine hydrochloride (4.5 mg L^{-1}) and kanamycin (50 mg L^{-1}). The components for feed contained glucose (734 g L^{-1}), $MgSO_4.7H_2O$ (20 g L^{-1}), EDTA (13 mg L^{-1}), trace elements (2.5 ml L^{-1}) and kanamycin (100 mg L^{-1}). Solution containing trace elements (400X) was composed of $CoCl_2$ $6H_2O$ (1 mg mL^{-1}), $MnCl_2$ $4H_2O$ (6 mg mL^{-1}), $CuCl_2$ $2H_2O$ (0.6 mg mL^{-1}), H_3BO_3 (1.2 mg mL^{-1}), Na_2MoO_4 $2H_2O$ (1 mg mL^{-1}), Zn (II) acetate $2H_2O$ (5.2 mg mL^{-1}) and Fe (III) citrate (40 mg mL^{-1}) [6]. The following initial set points, which were controlled through proportional-integral-derivative (PID) controller, were used for cultivation, pH: 6.8 ± 0.1, stirrer speed: 500 RPM, dissolved oxygen (DO): 100 ± 5%, air flow: 0.6 vvm, temperature: 37 °C. DO was maintained at 40% of air saturation by controlling the stirrer speed and aeration rate through the cascade controller. The maximum set values for stirrer speed and air flow were 1000 RPM and 2.0 vvm respectively. When the stirrer speed and air flow reached their maximum values the controller maintained the requirement of DO by pulsing pure oxygen through air enrichment module of fermenter.

It was observed that the yield coefficient ($Y_{x/s}$) was not constant during the process and decreased as fermentation progressed as reported previously [6]. Thus a correlation between glucose (S) vs. OD_{600} (Biomass produced) was derived from fermentation batches performed previously (internal lab data) with mass flow controllers by fitting second order polynomial as described in equation (1) below.

$$\text{Gulcose}(S_i) = 0.081(OD_i)^2 + 0.5314OD_i + 7.7876 \quad (1)$$

where S_i is the glucose required to generate the biomass (ODi) and was applied in preparation of feedback control to dispense the feed to the fermenter exponentially.

Feedback algorithm was created for exponential feeding by incorporating offline OD_{600} using equations (1)–(3).

$$m_s(i) = \left(\mu_{set} + mY_{x/s}\right)VS_i \quad (2)$$

$$r_s(t) = m_s(i)e^{\mu_{set}(t-t_i)} \quad (3)$$

where r_s (t) is the rate of addition of glucose (g/h). m_s(i) is value of