Available online at www.sciencedirect.com
ScienceDirect
Vaccine 25 (2007) 3713–3721
Vaccine
www.elsevier.com/locate/vaccine

The antigenicity of a *Plasmodium vivax* reticulocyte binding protein-1 (PvRBP1) recombinant fragment in humans and its immunogenicity and protection studies in *Aotus* monkeys

Jose Rojas Caraballo[a], Gabriela Delgado[a,b], Raul Rodriguez[a], Manuel A. Patarroyo[a,c,*]

[a] *Fundacion Instituto de Inmunologia de Colombia (FIDIC), Bogota, Colombia*
[b] *Immunotoxicology Research Group, Pharmacy Department, Universidad Nacional de Colombia, Bogota, Colombia*
[c] *Universidad Nacional de Colombia, Bogota, Colombia*

Received 29 September 2006; received in revised form 12 December 2006; accepted 18 December 2006
Available online 3 January 2007

Abstract

Plasmodium vivax merozoites have been found to specifically invade immature red blood cells (reticulocytes) and this preference has been associated with two proteins named reticulocyte binding protein-1 and protein-2 (PvRBP1 and PvRBP2). Previous reticulocyte binding assays using 15-mer synthetic peptides spanning the entire PvRBP1 sequence have shown that 25 out of the 195 peptides synthesised (grouped into 4 different regions) displayed high affinity binding to reticulocytes. The PvRBP1 region III (amino acids 1998–2348), encompassing 9 of the previously described high-affinity reticulocyte binding peptides, was expressed as a recombinant protein in the present study. This protein has been shown to be antigenic in humans and it has also been able to induce good humoral and cellular immune responses in *Aotus nancymaae* monkeys. Despite its high immunogenicity, no protective efficacy was observed in the immunised animals.

Keywords: *Plasmodium vivax*; Reticulocyte binding protein; Immune response; *Aotus* monkeys

1. Introduction

Malaria is a parasitic disease which is transmitted by female *Anopheles* mosquitoes and has been considered to be one of the most prevalent infectious diseases in the world. Around 300–500 million cases of malaria occur annually, more than 2 million being mortal [1–3]. Only 4 out of the close to 100 *Plasmodium* species affect humans (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*); *P. falciparum* and *P. vivax* are the most common, *P. falciparum* being more prevalent in Africa and *P. vivax* in Asia and the Americas [3,4].

P. vivax is responsible for around 50% of all cases of malaria occurring outside the African continent, mainly in Middle east, South-eastern Asia and South and Central America. Due to the foregoing and the parasite's increased resistance to anti-malarial drugs [5] as well as the vector's increased resistance to insecticides [6], the need for a vaccine against *Plasmodium* has become an urgent priority. However, progress in developing a vaccine which can effectively control *P. vivax* has been limited partly due to the lack of knowledge regarding vaccine candidates with respect to *P. falciparum*. Only partial information is available concerning *P. vivax* genome [7] and there is no data regarding its proteome and transcriptome. Only six new blood stage antigens have been described in *P. vivax* during the last 3 years: RAMA [8], PvTRAg [9], MSP8 [10], MSP10 [11], RAP-1 [12] and RAP-2 [13].

Abbreviations: RBP, reticulocyte binding protein; IPTG, isopropyl-1-thio-β-galactopyranoside; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; IFA, immunofluorescence antibody test; PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin M; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay

* Corresponding author at: Fundacion Instituto de Inmunologia de Colombia (FIDIC), Carrera 50# 26-00, Bogota, Colombia.
Tel.: +57 1 3244672x141; fax: +57 1 4815269.
E-mail address: mapatarr@fidic.org.co (M.A. Patarroyo).

0264-410X/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2006.12.041

One of *P. vivax* merozoites' main characteristics lies in its selective invasion of human reticulocytes and previously described reticulocyte binding proteins (RBP) 1 and 2 participate in this molecular event [14,15].

The *P. vivax* reticulocyte binding protein-1 (PvRBP1) is expressed in the merozoite's apical pole. It is a protein having high molecular weight (~325 kDa). It specifically bound to reticulocytes in an erythrocyte binding assay (EBA) described by Galinski et al. [15], since this protein had no binding activity when red blood cells (RBC) were reticulocyte-depleted and binding activity became significantly increased when this cell population was enriched with reticulocytes. PvRBP1 also has two RGD motifs which could have a reticulocyte binding function.

Urquiza et al. described four regions within the PvRBP1 sequence containing 25 high reticulocyte binding peptides [16]; the so-called region III, comprising amino acids (aa) 1998–2348, was the most conserved. It also contained nine high reticulocyte binding peptides which displayed greater binding activity than peptides from the other regions. Due to this protein's great importance in invasion of reticulocytes, and as it could be considered as a candidate for a vaccine against *P. vivax*, this region was expressed as a recombinant protein in *Escherichia coli* in the present study and its immunogenicity and protection-inducing ability were evaluated in *Aotus nancymaae* monkeys, as well as its antigenicity in humans.

2. Materials and methods

2.1. Expression and purification of rPvRBP1-reg III recombinant protein

The gene encoding the PvRBP1 region III was cloned in pQE-30 expression vector which adds six-histidine tag in the N-terminal region, thereby facilitating the protein being detected by anti-polyhistidine monoclonal antibodies and its subsequent purification. The recombinant plasmid was transfected in *E. coli* M15 bacteria.

Recombinant clones were then used for inoculating 50 mL of Terrific Broth culture medium (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 2.31 g/L KH_2PO_4, 12.54 g/L K_2HPO_4) containing ampicillin and kanamycin at 0.1 and 0.025 mg/mL final concentration, respectively. Following growth at 37 °C overnight, this inoculum was transferred to 1 L culture medium containing the same antibiotic concentrations and grown at 37 °C with constant shaking until reaching ~0.6 OD_{600}. Recombinant protein was expressed by adding isopropyl-1-thio-β-galactopyranoside (IPTG) at 1 mM final concentration for 5 h at 37 °C.

The bacterial pellet was recovered by centrifuging at 10,000 × *g* for 20 min and suspended in 0.1 M sodium phosphate, 6 M urea, 20 mM imidazole at 30 mL final volume. One milligram per milliliter of lysozyme was added to the suspension and sonicated for 1 min with a Branson digital sonifier (Branson, Los Angeles, USA), followed by centrifuging at 10,000 × *g* for 15 min; the pellet was then discarded.

The clear supernatant was applied to a Ni^{2+}-NTA resin according to manufacturer's recommendations [17]. Briefly, the resin was equilibrated with 0.1 M sodium phosphate, 6 M urea and 20 mM imidazole. The non-retained fraction was eluted with the same buffer and bound protein was eluted with 0.1 M sodium phosphate, 500 mM imidazole buffer. The fractions were collected and analysed by SDS-PAGE and Western blot.

2.2. SDS-PAGE and Western blot of rPvRBP1-reg III recombinant protein

The affinity chromatography fractions were separated by electrophoresis on 12% polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS) under reducing conditions and then transferred to nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS–0.05% Tween for 1 h at room temperature and washed three times with PBS–0.05% Tween. Anti-polyhistidine monoclonal antibody coupled to peroxidase was incubated at 1:5000 dilution for 2 h at room temperature. After washing the membrane thrice with PBS–0.05% Tween, it was revealed with a "VIP peroxidase substrate" kit (Vector Laboratories, Burlingame, Canada), according to manufacturer's recommendations.

The pure recombinant protein fractions were pooled and exhaustively dialysed against PBS 1×, pH 7.4, and concentrated by ultrafiltration; protein content was determined by bicinchoninic acid microassay [18], using bovine serum albumin (BSA) as standard.

2.3. A. nancymaae monkey immunisation

The animals used in this study were treated according to the conditions previously established by the Colombian Ministry of Health (governed by law 84/1989) and the Office for Protection from Research Risks (OPRR, Department of Health and Human Services, USA). The animals were supervised by a biologist specializing in primatology. Twenty *A. nancymaae* monkeys from the Colombian Amazon region which did not present evidence of prior *Plasmodium* infection as determined by immunofluorescence antibody test (IFA) at 1:20 dilution of their sera were used in this study.

A group of 10 monkeys was immunised with 50 μg rPvRBP1-reg III recombinant protein emulsified in Freund's complete adjuvant (FCA) on day 0; they were then immunised with the same amount of antigen in Freund's incomplete adjuvant (FIA) on days 20 and 40. Another group of 10 animals was used as control and only received PBS plus Freund's adjuvant, in the same conditions. The animals were bled on days 0, 20, 40 and 60 and their sera were assayed for detecting anti-rPvRBP1-reg III antibodies.

2.4. Challenge and parasitaemia assessment

Twenty days after the third immunization, both immunised and control *A. nancymaae* monkeys were intravenously challenged with 2.5×10^6 of the *P. vivax* VCG-1 strain asexual blood stage parasites taken from previously infected *A. nancymaae* monkey donors. After day 4, monkeys were followed-up daily for the development of parasitaemia by quantitative Giemsa-stained films and Acridine Orange staining determined by fluorescence microscopy. Non-protected monkeys were treated with 30 mg chloroquine administered over 2 days (15 mg per day) following the conclusion of the experiment (day 17), or when parasitaemia levels reached ≥6%. Afterwards, they were quarantined and examined for blood parasites to ensure cure and subsequently returned to the jungle.

2.5. rPvRBP1-reg III recognition by P. vivax-infected human sera and monkey sera using an ELISA assay

Polysorb (Nunc, Kamstrupvej, Denmark) plates were loaded with 1 μg recombinant protein per well, incubated overnight at 4 °C and washed thrice with PBS–0.05% Tween. The plates were blocked at 37 °C for 1 h with 100 μL 5% skimmed milk in PBS–0.05% Tween. The sera samples were added in duplicate in a 1:100 dilution and incubated for 1 h at 37 °C and plates were washed thrice with PBS–0.05% Tween. One hundred microliters of either a 1:8000 dilution of goat anti-human IgG or a 1:10,000 dilution of goat anti-*Aotus* IgG antibodies coupled to peroxidase were added as secondary antibodies. Following 1 h incubation at 37 °C, the excess of peroxidase-coupled antibody was removed by washing thrice with PBS–0.05% Tween. Plates were revealed by using a TMB Microwell Peroxidase Substrate System kit, following the manufacturer's instructions (KPL Laboratories, WA, USA). A Labsystems Multiskan MJ ELISA reader was used for reading absorbance at 620 nm (A_{620}). Monkey antibody titres were determined by successive two-fold primary antibody dilutions until reaching an A_{620} value equal to control value ±2S.D.

2.6. Detecting monkey anti-rPvRBP1-reg III antibodies by immunoblotting

The recombinant protein was size separated on a 12% polyacrylamide gel in the presence of SDS and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS–0.05% Tween for 1 h at room temperature, washed thrice with PBS–0.05% Tween and cut into strips for evaluating each monkey's serum. The pre-immune and immune sera from each monkey diluted 1:100 were incubated in a 5% skimmed milk-PBS–0.05% Tween solution for 1 h at room temperature, washed thrice with PBS–0.05% Tween and the peroxidase-coupled secondary antibody (goat anti-*Aotus* IgG), at 1:4000 dilution, was incubated for 1 h at room temperature. A "VIP Peroxidase substrate" kit (Vector Laboratories) was used for revealing the blots according to the manufacturer's recommendations.

2.7. Isolating PBMCs and lymphoproliferation assays

Peripheral blood mononuclear cells (PBMCs) isolated from *A. nancymaae* monkey 3 mL blood samples were obtained by gradient centrifugation in Ficoll-Hypaque (1.077 density) (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). 1×10^5 PBMCs per well were used as T-cell source. Ten micrograms per milliliter PvRBP1 recombinant protein was used as antigen. Phytohaemagglutinin M (PHA) (Difco, MI, USA) was used as positive cell proliferation control (5 μg/mL). Cell isolation was carried out in triplicate and cultured in 100 μl complete medium (RPMI-1640 supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium piruvate, 25 U/mL penicillin, 50 mg/mL streptomycin and 5×10^{-5} M 2-mercapto-ethanol and 10% autologous plasma). After 72 h culture at 37 °C and 5% CO_2 in air, the supernatant was harvested and 100 μL per well fresh complete medium, containing 1 μCi [^{3}H]-thymidine (Amersham-Pharmacia, Buckinghamshire, UK), were added to pulse the cultures for the last 16 h. The cells were then harvested and specific incorporation was determined by liquid scintillation in a beta counter. The results are expressed as stimulation index (SI), determined as being mean cpm in recombinant protein-stimulated culture/mean cpm in control cultures incubated without antigen. A SI higher than 3 represented a statistically significant difference.

2.8. Indirect immunofluorescence assay

P. vivax schizont-infected erythrocytes were separated from *A. nancymaae* monkeys' 3 mL blood samples by centrifuging using discontinuous Percoll gradient [19]. Twenty microliters of these schizont-parasitized samples were then sown in eight-well multi-test slides (ICN, Irvine, CA, USA); the sample was air-dried, fixed with 4% formaldehyde in PBS for 6 min at room temperature, briefly washed with PBS and then permeabilised with 0.2% Triton X-100 in PBS for 6 min. The slides were washed again twice with PBS. Each well was then blocked for 15 min with 5% BSA in PBS. *Aotus* immune sera directed against the recombinant protein were used as primary antibody at 1:100 dilution in PBS with 5% BSA. Fluorescein isothiocyanate (FITC) conjugated anti-*Aotus* IgG was used as secondary antibody at 1:40 dilution in PBS with 5% BSA. An Olympus BX551 fluorescence microscope was used for ascertaining immunofluorescence.

2.9. Statistical analysis

Monkey antibody titres were expressed as the mean ± two standard deviations. An unpaired *t*-test was used to assess significant differences amongst sera samples from *P. vivax*-infected patients who had suffered from a number of malarial episodes.

3. Results

3.1. Expression and purification of recombinant protein

An expression vector allowing the addition of a six histidine tag in the recombinant protein N-terminal extreme was selected as a tool for detecting and purifying the recombinant protein. This system led to obtaining an insoluble protein expressed in inclusion bodies. Denaturing conditions (6 M urea) were necessary for solubilising the protein of interest. Protein expression level was monitored by Western blot using monoclonal anti-polyhistidine antibodies. Initially, the recombinant protein was purified by affinity chromatography on Ni^{2+}-NTA resin coupled to agarose and the fractions so collected were analysed by SDS-PAGE and Western blot. Those fractions displaying a single band by SDS-PAGE and Western blot were collected and concentrated by ultrafiltration for obtaining the recombinant protein (Fig. 1), having a 48 kDa molecular weight. The protein was exhaustively dialysed against PBS 1× to ensure that the denaturing agent had been completely removed and that the protein had been obtained in a conformation as close to the native one as possible. The number of cysteines participating in forming disulphide bridges was determined by Ellman's assay [20], showing that three out of the three cysteines found within PvRBP1 region III were not forming disulphide bridges.

A band coinciding with the recombinant protein's molecular weight appeared in non-induced bacterial lysate; however, there was no recognition in this lysate by Western blot using anti-polyhistidine monoclonal antibody.

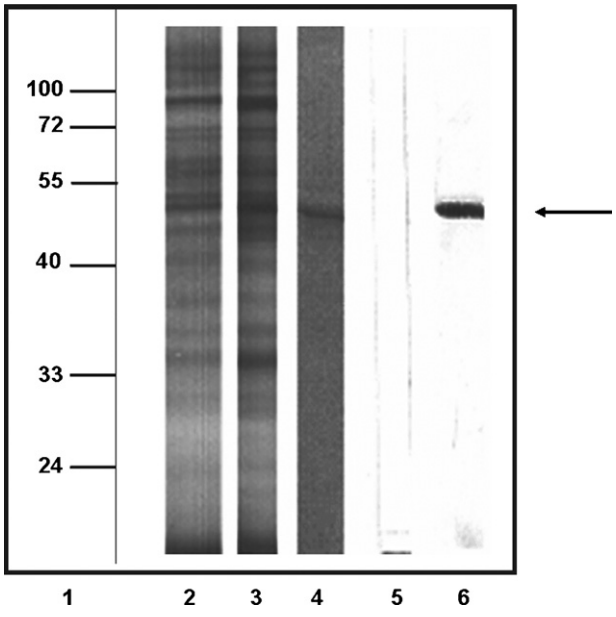

Fig. 1. SDS-PAGE and Western blot corresponding to purified rPvRBP1-reg III. Lane (1) Molecular weight markers (kDa), lane (2) non-induced bacterial lysate, lane (3) IPTG-induced bacterial lysate, lane (4) SDS-PAGE for the purified protein, lane (5) Western blot for the non-induced lysate and lane (6) Western blot for the purified protein using anti-polyhistidine monoclonal antibody.

3.2. rPvRBP1-reg III recombinant protein immunogenicity in A. nancymaae monkeys

3.2.1. Humoral immune response

Purified recombinant protein was formulated with Freund's adjuvant as previously described in Section 2 and used for immunising the *A. nancymaae* monkeys. As detected in Western blot and ELISA (Fig. 2a and b), a significant increase in antibody production could be observed following the second dose, which was maintained following the third immunisation. Antibody response was reproducible in the 10 monkeys and it can be seen in Fig. 2c that the titres obtained were high and presented values close to 1:51,200. However, a second band was observed in Fig. 2b which was attributed to protein degradation caused by prolonged storage and various freeze–thaw processes. Anti-rPvRBP1-reg III antibodies recognised the PvRBP1 protein in fresh *P. vivax* parasites by IFA (as shown in Fig. 3a) and in parasite lysate by Western blotting (Fig. 3b). The fluorescence pattern observed was unique, as expected for proteins localised in this region of the parasite.

3.2.2. Cellular immune response

The T-lymphocyte proliferation rendered in *A. nancymaae* monkeys previously immunised with the recombinant antigen was determined in this experiment, revealing appropriate antigen processing and T-cell presentation. Table 1 shows the data regarding the cellular proliferation of the 20 *A. nancymaae* monkeys included in this experiment; the monkeys were divided into two groups: those immunised with three doses of the recombinant antigen formulated in Freund's adjuvant and those inoculated with PBS 1× also formulated in Freund's adjuvant which were used as controls. T-lymphocyte stimulation with recombinant antigen became evident following the second dose and this response became boosted following the third one. No T-lymphocyte proliferation response was seen in the control group.

3.3. rPvRBP1-reg III recombinant protein protection-inducing ability in A. nancymaae monkeys

Although the recombinant protein displayed high immunogenicity, both at humoral and cellular levels, no protection was observed in vaccinated monkeys when challenged with the *P. vivax* VCG-1 strain. Monkeys belonging to both vaccinated and non-vaccinated groups displayed a similar behaviour reaching maximum parasitaemia between days 7 and 8. No significant differences regarding peak parasitaemia were observed in either group (Fig. 4a and b).

3.4. Recombinant protein antigenicity in humans

Sera from humans who had been living in endemic regions in Colombia (mainly coming from the eastern plains of the country (Guaviare and Norte de Santander)) and who had presented different episodes of *P. vivax* malaria were assayed

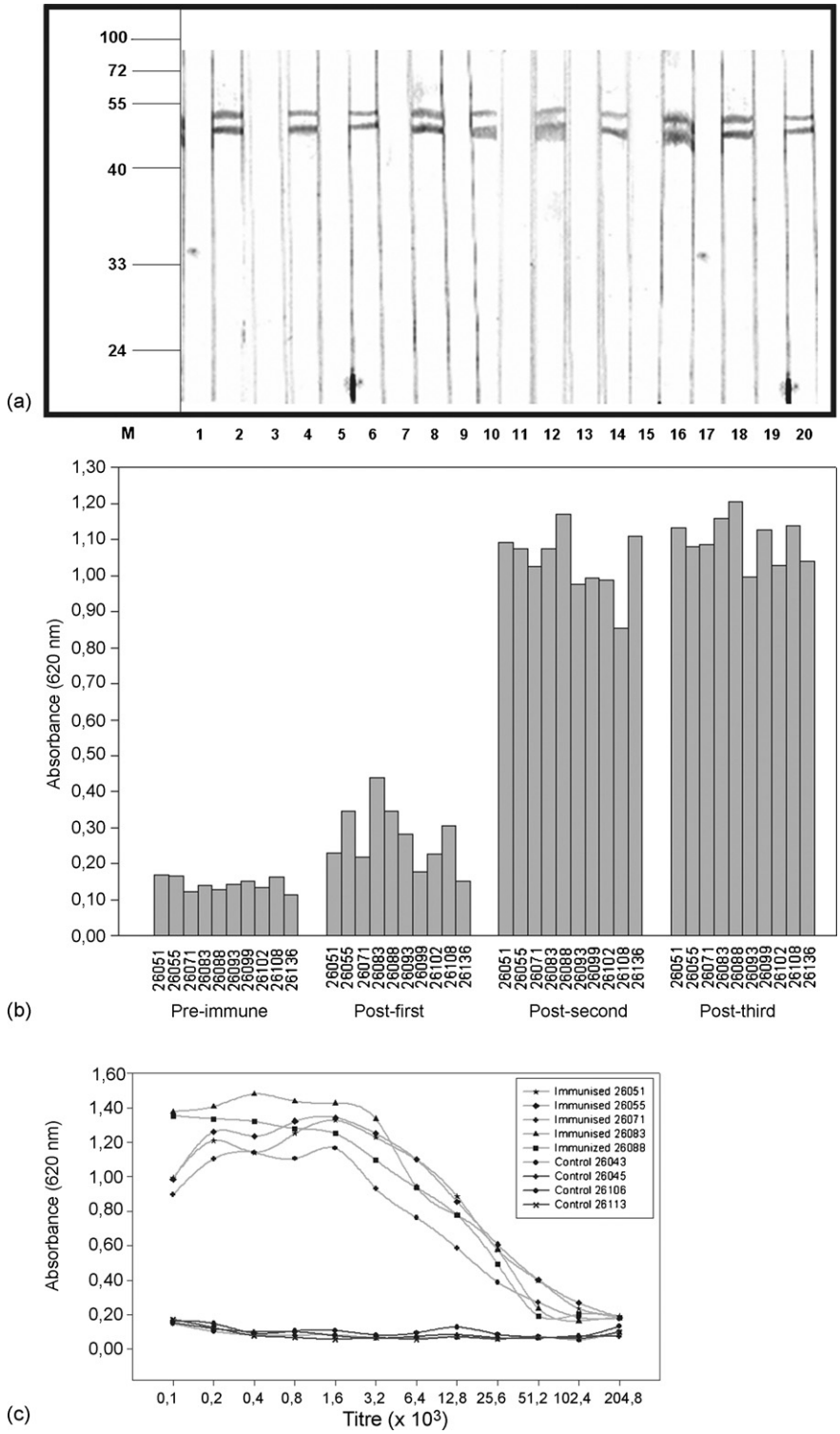

Fig. 2. Anti-rPvRBP1-reg III antibody production and detection by Western blot and ELISA. (a) Western blot corresponding to recombinant protein detection by all monkeys immunised with recombinant antigen. Oddly numbered lanes, pre-immune sera diluted 1:100; evenly numbered lanes, immune sera diluted 1:100 for all monkeys. (b) Immunised monkey antibody production following each recombinant antigen dose. (c) Determining anti rPvRBP1-reg III antibody titres. Two-fold dilutions of the sera were used; the titre value was determined as being pre-immune sera value ±2S.D.

Table 1
Lymphoproliferation assay using rPvRBP1-reg III as model antigen and PHA as mitogen

	Stimulation index (SI)							
	Pre-immune		Post-first		Post-second		Post-third	
	rPvRBP1-reg III	PHA	rPvRBP1-reg III	PHA	rPvRBP1-reg III	PHA	rPvRBP1-reg III	PHA
26051[a]	1	16	1	4	2	5	3	5
26055[a]	1	9	1	4	4	4	7	2
26071[a]	1	9	0	8	4	10	14	21
26083[a]	1	16	1	7	2	12	3	6
26088[a]	2	6	1	10	2	7	13	25
26093[a]	2	44	1	3	2	7	10	16
26099[a]	1	33	1	4	3	6	4	4
26102[a]	3	45	1	7	5	9	4	4
26108[a]	1	25	1	34	1	7	6	2
26136[a]	1	20	1	11	1	8	4	7
26043[b]	1	6	2	10	1	2	1	3
26045[b]	2	13	1	7	1	1	1	3
26106[b]	1	6	2	15	2	4	1	4
26113[b]	1	4	1	11	1	11	1	6
26116[b]	1	2	1	6	1	2	1	4
26118[b]	1	2	ND	ND	3	5	1	1
26121[b]	1	5	1	13	1	4	1	2
26122[b]	1	36	1	2	1	3	1	4
26126[b]	1	3	1	7	1	9	2	5
26089[b]	2	21	2	3	1	7	1	8

[a] Group of monkeys immunised with rPvRBP1-reg III.
[b] Group of monkeys used as control and immunised with PBS.

by ELISA; sera from patients from non-endemic areas were used as control. Fig. 5 shows that recombinant antigen recognition by sera increased with the number of malarial episodes suffered by the patients.

4. Discussion

RBPs have been previously described and characterised in *P. vivax* [14,15,21], being considered to be most important since they directly intervene in merozoites' specific binding to reticulocytes. *P. vivax* RBP homologue proteins have also been described in *P. falciparum* [22–26] and in other *Plasmodium* species such as *Plasmodium yoelli* [27], *Plasmodium cynomolgi* [28] and *Plasmodium reichenowi* [29].

It was decided to work with a recombinant segment of the PvRBP1 from the so-called region III due to this protein's great importance in reticulocyte invasion and the urgent need to find new antigens which might be included in an effective vaccine against *P. vivax*. Region III has been shown to have high reticulocyte binding ability, containing nine high binding activity peptides [16].

This recombinant antigen formulated in Freund's adjuvant was highly immunogenic in this study, inducing specific anti-rPvRBP1-reg III antibodies (Fig. 2a) with high antibody titres (1:51,200) (Fig. 2c) and T-lymphocyte proliferation (Table 1) in *A. nancymaae* monkeys, which lead us to suggest that the recombinant fraction used in the study specifically stimulated the cellular response, producing antigen-specific cellular clones which became expanded and evident in vitro when quantified by incorporating tritiated thymidine and compared to the same amount of cells but grown in the absence of antigen (which did not become divided due to the lack of specific stimulus: control). These antibodies were also able to recognise PvRBP1 in a fresh *P. vivax* preparation, revealing a unique fluorescence pattern, as expected for proteins located in this region of the parasite (Fig. 3a) [14] and also in a *P. vivax* lysate by using Western blot (Fig. 3b). High antibody titres have also been seen after inoculating other *P. vivax* blood stage recombinant antigens into *Saimiri boliviensis* monkeys [30]. Other *Plasmodium* blood stage recombinant antigens have also been shown to be immunogenic [31–36]. No significant difference has been observed in antibody production between the second and third immunisations; however, a significant difference in T-lymphocyte stimulation has been observed in cellular immune response during the same time. There was variability in proliferative response produced by *Aotus* monkeys regarding PHA mitogen in the control group; however, such heterogeneity has already been seen in *A. nancymaae* and *Aotus nigriceps* monkeys when using PHA-P mitogen [37].

PvRBP1-reg III recombinant protein antigenicity has also been shown here using sera from humans who have suffered several episodes of *P. vivax* malaria (Fig. 5), demonstrating that there is greater recognition when there has been an increased number of episodes. These results agree with those from studies carried out by Tran et al. [35] who showed *P. vivax* RBP1 antigenicity when using five recombinant fragments covering the whole protein sequence (aa 23–458, aa 431–748, aa 733–1047, aa 1392–2076 and aa 2038–2611).

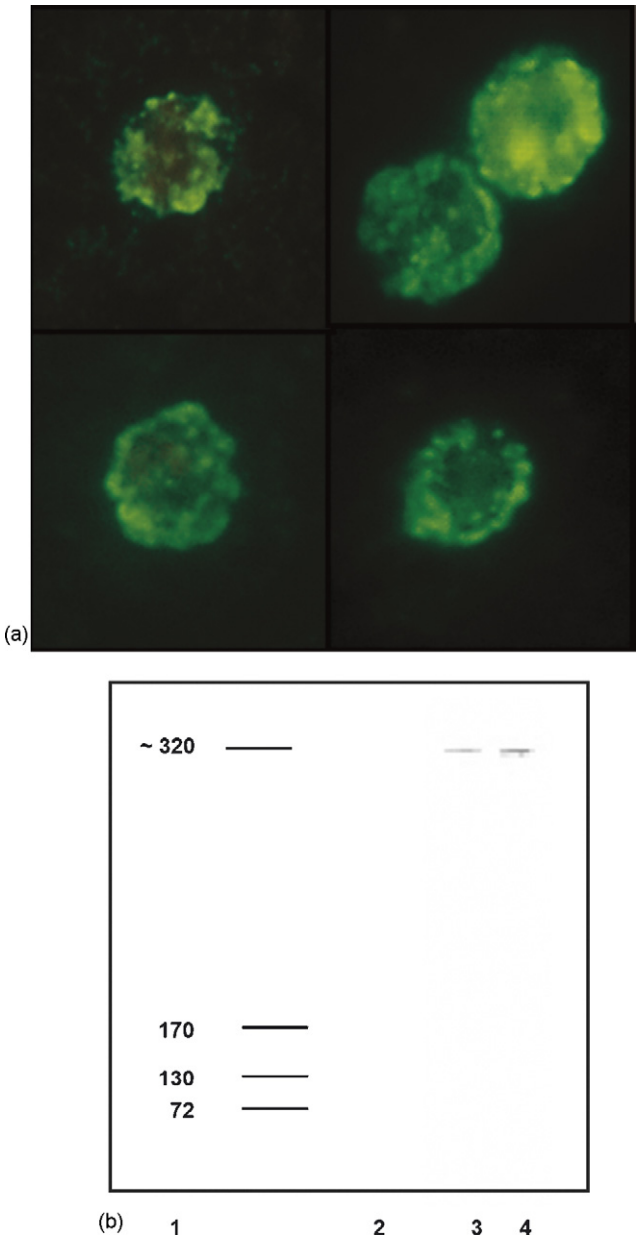

Fig. 3. PvRBP1 detection in *P. vivax* fresh parasite. (a) Indirect immunofluorescence assay using immune *Aotus* monkey's serum against rPvRBP1-reg III. A similar pattern was observed when other immune monkey sera were tested (data not shown). (b) PvRBP1 detected by Western blot in *P. vivax* lysate. Lane (1) Molecular weight marker (kDa), lane (2) preimmune serum and lanes (3 and 4) immune sera from monkeys 26051 and 26055, respectively.

However, they did find that the most antigenic segments corresponded to amino acids 433–748 and 733–1407 sequences containing 1 and 3 high reticulocyte binding ability peptides (HABPs), respectively, compared to the nine from our recombinant antigen, and that antigenic response increased with the time spent residing in the endemic area.

In spite of the recombinant protein inducing high immunogenicity, no protective efficacy was observed in the animal model being studied (Fig. 4a and b). Lack of protection could

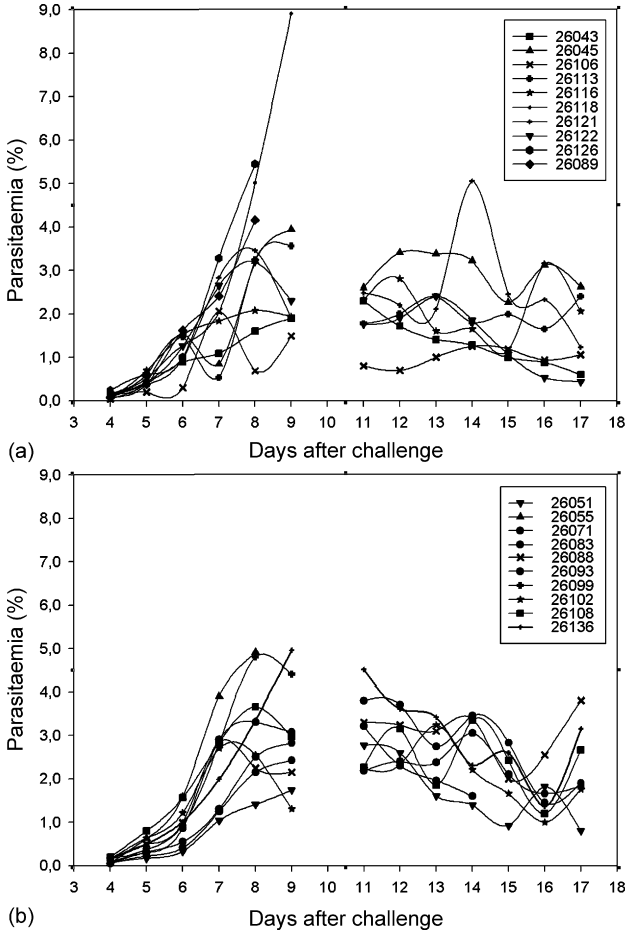

Fig. 4. Parasitaemia curves developed by *A. nancymaae* monkeys used in this study. (a) Control group of monkeys inoculated with PBS formulated in Freund's adjuvant. (b) Group of monkeys inoculated with recombinant antigen formulated in Freund's adjuvant.

be attributed to several aspects including: the need for structural configuration depending on the formation of disulphide bonds between this region and the rest of the protein or the formation of disulphide bonds with PvRBP2 [15], given that PvRBP1 contains 16 cysteines in its amino acid sequence which could be participating in forming intercatenary and

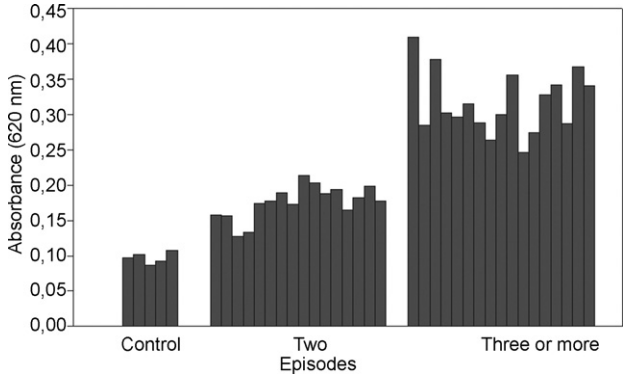

Fig. 5. ELISA for evaluating rPvRBP1-reg III antigenicity. The sera were used at 1:100 dilution.

intracatenary bridges; however, it is not known which cysteines participate in this event [14]. The rPvRBP1 region III contains three cysteines in its amino acid sequence (positions 2066, 2175 and 2208 in the Sal-I sequence) which do not participate in forming disulphide bridges according to Ellman's assay [20]. No evidence has been presented in our assays regarding any difference in rPvRBP1 region III mobility on SDS-PAGE gel, irrespective of whether DTT had been included (data not shown).

The need for modifying the protein's amino acid sequence should also be taken into account as it could be silent, as shown in prior studies with synthetic peptides derived from various *P. falciparum* antigens such as MSP1 [38,39], EBA-175 [40,41], RESA [42], ABRA [43] having similar characteristics to rPvRBP1-reg III as they are conserved and display high binding ability to their target cells. They are non-immunogenic and non-protective in their native configuration; however, when chemical modifications are made to some of their amino acids, these analogues become immunogenic and protection inducing.

Evidence of partial protection against *P. vivax* has been observed when *A. nancymaae* monkeys previously immunised with recombinant fragments comprising most of the MSP1 33 kDa cleavage product including amino acids 1317–1425 ($rPvMSP1_{20}$) and 1554–1624 ($rPvMSP1_{14}$) formulated in Freund's adjuvant [33] were challenged with this parasite species, suggesting that protection could be mediated by antibody and IFN-γ production [44]. Protection has also been shown in *S. boliviensis* monkeys with another PvMSP1 recombinant segment belonging to MSP1's C-terminal fragment (aa 1622–1729) [30].

The results obtained in this study have shown PvRBP1-reg III recombinant protein's high immunogenicity in *A. nancymaae* monkeys and its antigenicity in humans who have suffered one or more infections by *P. vivax*. Our goal when starting the present study was to evaluate the potential protective efficacy rendered by this recombinant protein in order to combine it (in a future work) with the previously studied $rPvMSP1_{20}$ and $rPvMSP1_{14}$ fragments to enhance their protective efficacy in a multi-antigen blood stage vaccine candidate. However, since no protection in monkeys immunised with PvRBP1-reg III alone was observed, we do not think the inclusion of the latter, in its present form, to the mixture could improve protection.

Acknowledgements

We would like to thank Jason Garry and Tricia Hadley for reviewing the manuscript and Jefferson Medina and Jimena Cortes for their technical support. We would also like to thank Professor Manuel Elkin Patarroyo for his invaluable comments and suggestions. This work was supported by the President of Colombia's office and the "Instituto Colombiano para el Desarrollo de la Ciencia 'Francisco Jose de Caldas' (Colciencias)" Contract #RC-0060-2006.

References

[1] Greenwood B, Mutabingwa T. Malaria in 2002. Nature 2002;415:670–2.
[2] Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature 2005;434:214–7.
[3] Suh KN, Kain KC, Keystone JS. Malaria. CMAJ 2004;170:1693–702.
[4] Mendis K, Sina BJ, Marchesini P, Carter R. The neglected burden of *Plasmodium vivax* malaria. Am J Trop Med Hyg 2001;64:97–106.
[5] Ridley RG. Medical need, scientific opportunity and the drive for antimalarial drugs. Nature 2002;415:686–93.
[6] Chandre F, Darrier F, Manga L, Akogbeto M, Faye O, Mouchet J, et al. Status of pyrethroid resistance in *Anopheles gambiae sensu lato*. Bull World Health Organ 1999;77:230–4.
[7] Carlton J. The *Plasmodium vivax* genome sequencing project. Trends Parasitol 2003;19:227–31.
[8] Topolska AE, Black CG, Coppel RL. Identification and characterisation of RAMA homologues in rodent, simian and human malaria species. Mol Biochem Parasitol 2004;138:237–41.
[9] Jalah R, Sarin R, Sud N, Alam MT, Parikh N, Das TK, et al. Identification, expression, localization and serological characterization of a tryptophan-rich antigen from the human malaria parasite *Plasmodium vivax*. Mol Biochem Parasitol 2005;142:158–69.
[10] Perez-Leal O, Sierra AY, Barrero CA, Moncada C, Martinez P, Cortes J, et al. *Plasmodium vivax* merozoite surface protein 8 cloning, expression, and characterisation. Biochem Biophys Res Commun 2004;324:1393–9.
[11] Perez-Leal O, Sierra AY, Barrero CA, Moncada C, Martinez P, Cortes J, et al. Identifying and characterising the *Plasmodium falciparum* merozoite surface protein 10 *Plasmodium vivax* homologue. Biochem Biophys Res Commun 2005;331:1178–84.
[12] Perez-Leal O, Mongui A, Cortes J, Yepes G, Leiton J, Patarroyo MA. The *Plasmodium vivax* rhoptry-associated protein 1. Biochem Biophys Res Commun 2006;341:1053–8.
[13] Patarroyo MA, Perez-Leal O, Lopez Y, Cortes J, Rojas-Caraballo J, Gomez A, et al. Identification and characterisation of the *Plasmodium vivax* rhoptry-associated protein 2. Biochem Biophys Res Commun 2005;337:853–9.
[14] Galinski MR, Barnwell JW. *Plasmodium vivax*: merozoites, invasion of reticulocytes and considerations for malaria vaccine development. Parasitol Today 1996;12:20–9.
[15] Galinski MR, Medina CC, Ingravallo P, Barnwell JW. A reticulocyte-binding protein complex of *Plasmodium vivax* merozoites. Cell 1992;69:1213–26.
[16] Urquiza M, Patarroyo MA, Mari V, Ocampo M, Suarez J, Lopez R, et al. Identification and polymorphism of *Plasmodium vivax* RBP-1 peptides which bind specifically to reticulocytes. Peptides 2002;23:2265–77.
[17] Qiagen. The QIAexpressionist. A handbook for high-level expression and purification of 6xhis-tagged proteins. 5th ed. Valencia, CA: Qiagen; 2003.
[18] Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. Measurement of protein using bicinchoninic acid. Anal Biochem 1985;150:76–85.
[19] Andrysiak PM, Collins WE, Campbell GH. Concentration of *Plasmodium ovale*- and *Plasmodium vivax*-infected erythrocytes from nonhuman primate blood using Percoll gradients. Am J Trop Med Hyg 1986;35:251–4.
[20] Creighton TE. Disulphide bonds between cysteine residues. In: Creigton TE, editor. Protein structure—a practical approach. Oxford: IRL Press; 1995. p. 157.
[21] Cantor EM, Lombo TB, Cepeda A, Espinosa AM, Barrero CA, Guzman F, et al. *Plasmodium vivax*: functional analysis of a highly conserved PvRBP-1 protein region. Mol Biochem Parasitol 2001;117:229–34.
[22] Kaneko O, Mu J, Tsuboi T, Su X, Torii M. Gene structure and expression of a *Plasmodium falciparum* 220-kDa protein homologous to the *Plas-*

modium vivax reticulocyte binding proteins. Mol Biochem Parasitol 2002;121:275–8.

[23] Ocampo M, Vera R, Rodriguez LE, Curtidor H, Suarez J, Garcia J, et al. Identification of *Plasmodium falciparum* reticulocyte binding protein RBP-2 homologue a and b (PfRBP-2-Ha and -Hb) sequences that specifically bind to erythrocytes. Parasitol Int 2004;53: 77–88.

[24] Rayner JC, Galinski MR, Ingravallo P, Barnwell JW. Two *Plasmodium falciparum* genes express merozoite proteins that are related to *Plasmodium vivax* and *Plasmodium yoelii* adhesive proteins involved in host cell selection and invasion. Proc Natl Acad Sci USA 2000;97: 9648–53.

[25] Singh S, Miura K, Zhou H, Muratova O, Keegan B, Miles A, et al. Immunity to recombinant *Plasmodium falciparum* merozoite surface protein 1 (MSP1): protection in *Aotus nancymai* monkeys strongly correlates with anti-MSP1 antibody titer and in vitro parasite-inhibitory activity. Infect Immun 2006;74:4573–80.

[26] Triglia T, Thompson J, Caruana SR, Delorenzi M, Speed T, Cowman AF. Identification of proteins from *Plasmodium falciparum* that are homologous to reticulocyte binding proteins in *Plasmodium vivax*. Infect Immun 2001;69:1084–92.

[27] Galinski MR, Xu M, Barnwell JW. *Plasmodium vivax* reticulocyte binding protein-2 (PvRBP-2) shares structural features with PvRBP-1 and the *Plasmodium yoelii* 235 kDa rhoptry protein family. Mol Biochem Parasitol 2000;108:257–62.

[28] Okenu DM, Meyer EV, Puckett TC, Rosas-Acosta G, Barnwell JW, Galinski MR. The reticulocyte binding proteins of *Plasmodium cynomolgi*: a model system for studies of *P. vivax*. Mol Biochem Parasitol 2005;143:116–20.

[29] Rayner JC, Huber CS, Galinski MR, Barnwell JW. Rapid evolution of an erythrocyte invasion gene family: the *Plasmodium reichenowi* reticulocyte binding like (RBL) genes. Mol Biochem Parasitol 2004;133:287–96.

[30] Collins WE, Kaslow DC, Sullivan JS, Morris CL, Galland GG, Yang C, et al. Testing the efficacy of a recombinant merozoite surface protein (MSP-1(19)) of *Plasmodium vivax* in *Saimiri boliviensis* monkeys. Am J Trop Med Hyg 1999;60:350–6.

[31] Ahlborg N, Ling IT, Howard W, Holder AA, Riley EM. Protective immune responses to the 42-kilodalton (kDa) region of *Plasmodium yoelii* merozoite surface protein 1 are induced by the C-terminal 19-kDa region but not by the adjacent 33-kDa region. Infect Immun 2002;70:820–5.

[32] Nogueira PA, Alves FP, Fernandez-Becerra C, Pein O, Santos NR, Pereira da Silva LH, et al. A reduced risk of infection with *Plasmodium vivax* and clinical protection against malaria are associated with antibodies against the N terminus but not the C terminus of merozoite surface protein 1. Infect Immun 2006;74:2726–33.

[33] Sierra AY, Barrero CA, Rodriguez R, Silva Y, Moncada C, Vanegas M, et al. Splenectomised and spleen intact *Aotus* monkeys' immune response to *Plasmodium vivax* MSP-1 protein fragments and their high activity binding peptides. Vaccine 2003;21:4133–44.

[34] Stowers A, Taylor D, Prescott N, Cheng Q, Cooper J, Saul A. Assessment of the humoral immune response against *Plasmodium falciparum* rhoptry-associated proteins 1 and 2. Infect Immun 1997;65:2329–38.

[35] Tran TM, Oliveira-Ferreira J, Moreno A, Santos F, Yazdani SS, Chitnis CE, et al. Comparison of IgG reactivities to *Plasmodium vivax* merozoite invasion antigens in a Brazilian Amazon population. Am J Trop Med Hyg 2005;73:244–55.

[36] Yazdani SS, Shakri AR, Mukherjee P, Baniwal SK, Chitnis CE. Evaluation of immune responses elicited in mice against a recombinant malaria vaccine based on *Plasmodium vivax* Duffy binding protein. Vaccine 2004;22:3727–37.

[37] Pinzon-Charry A, Vernot JP, Rodriguez R, Patarroyo ME. Proliferative response of peripheral blood lymphocytes to mitogens in the owl monkey *Aotus nancymae*. J Med Primatol 2003;32:31–8.

[38] Espejo F, Bermudez A, Torres E, Urquiza M, Rodriguez R, Lopez Y, et al. Shortening and modifying the 1513 MSP-1 peptide's alpha-helical region induces protection against malaria. Biochem Biophys Res Commun 2004;315:418–27.

[39] Torres MH, Salazar LM, Vanegas M, Guzman F, Rodriguez R, Silva Y, et al. Modified merozoite surface protein-1 peptides with short alpha helical regions are associated with inducing protection against malaria. Eur J Biochem 2003;270:3946–52.

[40] Bermudez A, Cifuentes G, Guzman F, Salazar LM, Patarroyo ME. Immunogenicity and protectivity of *Plasmodium falciparum* EBA-175 peptide and its analog is associated with alpha-helical region shortening and displacement. Biol Chem 2003;384:1443–50.

[41] Cifuentes G, Salazar LM, Vargas LE, Parra CA, Vanegas M, Cortes J, et al. Evidence supporting the hypothesis that specifically modifying a malaria peptide to fit into HLA-DRbeta1*03 molecules induces antibody production and protection. Vaccine 2005;23:1579–87.

[42] Alba MP, Salazar LM, Vargas LE, Trujillo M, Lopez Y, Patarroyo ME. Modifying RESA protein peptide 6671 to fit into HLA-DRbeta1* pockets induces protection against malaria. Biochem Biophys Res Commun 2004;315:1154–64.

[43] Salazar LM, Alba MP, Curtidor H, Bermudez A, Luis EV, Rivera ZJ, et al. Changing ABRA protein peptide to fit into the HLA-DRbeta1*0301 molecule renders it protection-inducing. Biochem Biophys Res Commun 2004;322:119–25.

[44] Barrero CA, Delgado G, Sierra AY, Silva Y, Parra-Lopez C, Patarroyo MA. Gamma interferon levels and antibody production induced by two PvMSP-1 recombinant polypeptides are associated with protective immunity against *P. vivax* in *Aotus* monkeys. Vaccine 2005;23:4048–53.