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Immunogenicity and protection-inducing ability of recombinant *Plasmodium vivax* rhoptry-associated protein 2 in *Aotus* monkeys: A potential vaccine candidate

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ABSTRACT

Rhoptry proteins have been extensively shown to be important in invasion and parasitophorous vacuole (PV) formation. This work evaluates the immunogenicity and protective efficacy of *Plasmodium vivax* RAP2 in the non-human *Aotus* primate model, when expressed as a recombinant molecule in *E. coli* and formulated in Freund and Alum hydroxide adjuvants. Our results show that r*Pv*RAP2 is immunogenic in both formulations, finding a trend of higher cytokine levels in immunized monkeys, specially in IL-4 levels (using Freund's adjuvant) and IL-5 (using Alum hydroxide). RAP2 is suggested as a *P. vivax*-vaccine candidate since immunized monkeys exhibited lower parasitemias than control groups after being experimentally challenged with the *P. vivax* VCG-I strain.

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1. Introduction

Malaria is one of the most prevalent parasitic diseases among tropical countries. Every year nearly 500 million new cases are reported worldwide, of which an estimated 1–2 million result in death [1]. Epidemiologically speaking, *Plasmodium vivax* is considered as the second most important malarial parasite species, as it accounts for more than 75 million annual cases of malaria occurring mainly in Asia, Central and South America [2,3].

Despite the great efforts in developing an effective strategy for the control of this scourging disease, malaria remains a public health threat worldwide [4]. One of the most appealing strategies for eradicating this disease is the development of a vaccine. Nevertheless, obtaining a fully effective vaccine has been hindered by the great diversity of surface proteins expressed throughout the parasite's life cycle, its polymorphism and the fact that immunity induced by a natural infection is gradually acquired and species-, stage- and strain-specific [5–7]. Aiming at developing a vaccine against *P. falciparum*, several research groups have characterized new antigens in this species, among which the rhoptry-associated protein 2 (RAP2) has long been considered a major vaccine candidate due to its ability to induce antibodies capable of inhibiting *in vitro* invasion of red blood cells (RBCs) [8], its low degree of genetic polymorphism [9], its binding ability to RBCs [10], its recognition by sera from patients living in endemic areas [11] and, more importantly, its capacity to confer protection against experimental challenge with *P. falciparum* in *Saimiri boliviensis* monkeys [12]. All this evidence has encouraged its recent characterization in *P. vivax* [13], even though its role in RBCs invasion is still not clearly elucidated in this parasite species.

Diverse components of the immune system are involved in the response against *Plasmodium*, depending on the parasite's life stage. In the liver stage, specific parasite antigens are recognized by Interferon-γ (IFN-γ)-secreting CD8+ T lymphocytes [14]. IFN-γ-activated macrophages display a direct antiparasitic effect by secreting nitric oxide (NO) through an inducible nitric oxide synthase (iNOS) [15]. Regarding the erythrocytic stage, antibodies directed against merozoite surface antigens are able to block RBC invasion and/or facilitate phagocytosis [16,17]. Both Th1 and

Abbreviations: RAP2, rhoptry-associated protein 2; r*Pv*RAP2, recombinant rhoptry-associated protein 2; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBMC, peripheral blood mononuclear cells; ELISA, enzyme-linked immunosorbent assay; VCG-I, Vivax Colombia Guaviare I; PHA, phytohemagglutinin; RBCs, red blood cells; iRBCs, infected red blood cells.

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Th2 responses are directly involved in conferring immune protection against malarial blood stages and the balance between both cytokine profiles is important to determine disease outcome [17].

Given the difficulties in analyzing the human immune response, various animal models have been used to determine the mechanisms underlying the induction of protection against malaria, among which the World Health Organization (WHO) has suggested *Aotus* spp. monkeys as the ideal experimental non-human primate model for evaluating malaria vaccine candidates [18]. The aim of the present study was to evaluate the immunogenicity of the recombinant *Pv*RAP2 (r*Pv*RAP2) when it was formulated in either Freund's adjuvant or Alum hydroxide, as well as the resulting cytokine profile response and its protection-inducing ability in *Aotus nancymaae* monkeys against an experimental challenge with *P. vivax*.

2. Materials and methods

2.1. *P. vivax purification*

The *P. vivax* (Vivax-Colombia-Guaviare I (VCG-I)) strain was used as RNA and protein source. *P. vivax*-infected RBCs (iRBCs) were isolated from a 3 mL blood sample taken from an infected monkey using a 30–50% Percoll density gradient (Amersham Biosciences, Uppsala, Sweden) following a previously described protocol [19].

2.2. *Cloning and sequencing*

One microgram of total parasite RNA extracted by the Trizol method [20] and treated with RQ1 RNase-free DNase (Promega, Wisconsin, USA) was used as template for cDNA synthesis, using the SuperScript III enzyme (Invitrogen, California, USA) in 20 μL RT-PCR reactions carried out at 50 °C for 60 min, according to manufacturer's recommendations. The *Pv*RAP2 gene was then amplified in 35 PCR-cycles as follows: 15 s at 94 °C, 30 s at 56 °C, 80 s at 68 °C and a final 5 min extension at 68 °C; using Platinum *Pfx* DNA polymerase enzyme (Invitrogen, California, USA) and primers specifically designed to cover the entire *Pv*RAP2 transcript (5′-ATGCATACTTGCACAAAAGAATTG-3′ forward primer and 5′-TGACTCCATACCTTTCTCC-3′ reverse primer), according to the reported gene sequence [13]. PCR products were purified with the Wizard PCR preps kit (Promega, Wisconsin, USA) and cloned into pEXP5-CT/TOPO vector (Invitrogen, California, U SA), which adds a six-histidine tag at the C-terminus of the recombinant protein to allow its detection by anti-polyhistidine monoclonal antibodies and further purification by affinity chromatography. The resulting recombinant DNA plasmid was purified using a Miniprep purification system kit (Promega, Wisconsin, USA) and then sequenced in an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, California, USA) to verify the cloned insert integrity.

2.3. *rPvRAP2 expression and purification*

E. coli BL21 cells harboring the pEXP5-CT/TOPO recombinant construct were grown in 50 mL of Terrific broth (TB) 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, 0.1 mg/mL Ampicillin and 0.1% (w/v) D-glucose) for 12 h at 37 °C, under constant shaking. This culture was then used for inoculating 950 mL of TB medium, which was kept at 37 °C until reaching an optical density (OD_{600}) of 0.6–0.8. Once achieving this optimum cell concentration (after to 2 h approximately), L-arabinose was added at a 0.2% (w/v) final concentration to induce expression of the recombinant protein. Cells were further incubated at 37 °C for 5 h and then harvested by centrifugation at 12,000 × *g* for 30 min at 4 °C.

Protein solubilization was achieved using high concentrations of denaturing agents (6 M Urea, 10 mM Tris–Cl, 100 mM NaH_2PO_4 and 15 mM Imidazole), 1 mg/mL lysozyme and further sonication of the cellular pellet. The resulting recombinant solubilized protein was recovered from the supernatant by centrifugation at 12,000 × *g* for 30 min at 4 °C and its expression was verified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using anti-polyhistidine monoclonal antibodies. A Ni^{+2}-NTA Agarose resin was then used to purify r*Pv*RAP2 from the clear supernatant by affinity chromatography (Qiagen, CA). Briefly, the resin's pH was first adjusted with extraction buffer (6 M Urea, 10 mM Tris–Cl, 100 mM NaH_2PO_4 and 15 mM Imidazole) to pH 8 and the protein extract was then allowed to pass through the column. Non-retained proteins were eluted using the same buffer solution, while a lysis buffer containing 500 mM Imidazole was used to elute the recombinant protein. All fractions individually collected were analyzed by SDS-PAGE and Western blotting.

2.4. *Ascertaining the expression of rPvRAP2 by Western blotting*

The affinity chromatography fractions were separated by electrophoresis on 12% polyacrylamide gels in the presence of SDS (sodium-dodecyl sulphate) under reducing conditions and then transferred to a nitrocellulose membrane; using a 5% skimmed milk in PBS–0.05% Tween to block membranes for 1 h. After washing membranes thrice with PBS–0.05% Tween, they were incubated with peroxidase-coupled anti-polyhistidine monoclonal antibodies diluted 1:4500 in PBS–0.05% Tween containing 5% skimmed milk, for 2 h at room temperature; then washed thrice with PBS–0.05% Tween and revealed using the VIP Peroxidase substrate kit (Vector Laboratories, Burlingame, CAN) according to the manufacturer's recommendations. Pure fractions were pooled and dialyzed thoroughly against a step Urea gradient (6–1 M) to avoid protein precipitation. In brief, the protein was first dialyzed against 4 M Urea–PBS for 6 h, 2 M Urea–PBS for another 6 h, followed by dialysis against 1 M Urea–PBS and finally, thorough dialyzed against 1× PBS, pH 7.2. The protein was then ultrafiltrated and its concentration was determined by the bicinchoninic acid assay using BSA as standard [21].

2.5. *Immunization of A. nancymaae monkeys with rPvRAP2*

The immunization trials were carried out in *A. nancymaae* monkeys native to the Colombian Amazon region and kept in our Institute's primate station according to the guidelines stipulated by the Colombian Ministry of Health (law 84/1989) and the Office for Protection from Research Risks (OPRR, Department of Health and Human Services, USA), under the supervision of a primatologist. A total of 22 monkeys presenting no evidence of previous *Plasmodium* spp. infection, as determined by indirect immunofluorescence assay (IFA), were randomly distributed into immunization groups as follows. Two groups containing seven monkeys each, were immunized with 50 μg of recombinant antigen, the first one formulated in Freund's adjuvant and the second one in Alum hydroxide. Two groups of four monkeys were inoculated at the same immunization schedule with PBS 1× formulated in Freund's adjuvant and Alum hydroxide, respectively, and served as adjuvant controls. All animals received booster immunizations with their respective adjuvants on days 20 and 40; and were bled on days 0, 20, 40 and 60. The so obtained sera samples were used for evaluating the antibody titers.

2.6. *Challenge and parasitemia assessment*

Twenty days after the third immunization, all *A. nancymaae* monkeys were intravenously challenged with 2.5×10^6 of *P. vivax* VCG-I strain–iRBCs taken from previously infected *A. nancymaae* monkey donors. Beginning on day 4, monkeys were followed-

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