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Short communication

Vaccination with recombinant *Plasmodium vivax* MSP-10 formulated in different adjuvants induces strong immunogenicity but no protection

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ABSTRACT

Although largely considered benign, *Plasmodium vivax* causes disease in nearly 75 million people each year and the available strategies are not sufficient to reduce the burden of disease, therefore pointing to vaccine development as a cost-effective control measure. In this study, the *P. vivax* merozoite surface protein 10 (MSP-10) was expressed as a recombinant protein in *Escherichia coli* and purified by affinity chromatography. High antigenicity was observed since sera from *P. vivax*-infected patients strongly recognized rPvMSP10. The immunogenicity of rPvMSP10 was tested in *Aotus* monkeys, comparing responses induced by formulations with Freund's adjuvant, Montanide ISA720 or aluminum hydroxide. All formulations produced high antibody titers recognizing the native protein in late schizonts. Despite inducing strong antibody production, none of the formulations protected immunized *Aotus* monkeys upon experimental challenge.

1. Introduction

Malaria is one of the most serious public health problems worldwide. Nearly 50% of the world's population lives in endemic areas and according to the WHO, there were around 247 million cases of malaria and about 881,000 deaths in 2006 as a consequence of this disease [1,2]. The disease is caused by parasites of the genus *Plasmodium* and transmitted by the bite of the female *Anopheles* mosquito. Among the four genera causing malaria to humans, *Plasmodium falciparum* and *Plasmodium vivax* account for nearly 90% of all reported cases of malaria, being *P. falciparum* the one producing the most severe clinical cases in sub-Saharan Africa [1,3].

Even though malaria caused by *P. vivax* is rarely mortal, it causes around 75 million clinical episodes per year, usually distributed in Southern Asia and Western Pacific with a significant number occurring South America, which has placed this species as an important infectious agent. Efforts to control and prevent *P. vivax* malaria are far from achieving disease erradication due to the spread of parasite's resistance to the most common antimalarial drugs and of mosquitoes to insecticides used in its control, thus emphasizing the urgent need of identifying and characterizing potential vaccine candidates in this species [4].

The recent publication of the complete *P. vivax* genome and the transcriptome of its intraerythrocytic developmental cycle have opened an interesting pathway for the identification of new antigens [5,6], which were until recently carried out based on homology to important *P. falciparum* vaccine candidate proteins involved in invasion to red blood cells (RBCs), due to the intrinsic difficulties of maintaining a continuous *in vitro* culture of this species. A considerable number of antigens were identified by our group with such strategy, including RAP-2 [7], RAP-1 [8], PvMSP8 [9], PvRhopH3 [10], Pv41 [11], Pv38 [12], PvMSP7 [13] and PvMSP10 [14].

The family of merozoite surface proteins (MSPs) have been widely studied in different *Plasmodium* species due to its involvement in the initial steps of RBC invasion [15,16]. Among the members of this family, PvMSP10 was identified as a 479-amino acid polypeptide with an estimated molecular weight of 52 kDa. PvMSP10 is predicted to contain a signal sequence to the N-terminus and a glycosylphosphatidylinositol (GPI)-anchor as well as two Epidermal Growth Factor (EGF)-like domains localized to the C-terminus [14]. Moreover, it has been shown that antibodies directed against EGF-like domains inhibit parasite growth *in vitro* and that naturally acquired anti-EGF-like domain antibodies are

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associated with clinical immunity against *P. falciparum*, altogether suggesting the involvement of these domains in parasite invasion to host cells [17].

Based on the homology of PvMSP10 to PfMSP10 and its potential implication in RBC invasion, the aim of the present study was to assess the antigenicity, immunogenicity and protective efficacy of recombinant PvMSP10 (rPvMSP10) against experimental challenge in *Aotus* monkeys, when being administered with either aluminum hydroxide, Montanide ISA720 or Freund's adjuvant.

2. Materials and methods

2.1. Recombinant protein expression and purification

The gene encoding MSP-10 in *P. vivax* was cloned in the pQE-30 expression vector, which adds a polyhistidine (6-His) tag at the protein's N-terminus to facilitate further purification. Transformed *Escherichia coli* Rosetta-gami (RG) bacteria were cultured in 50 mL of Terrific Broth (TB) (12 g/L triptone, 24 g/L yeast extract, 4 mL/L glycerol, 2.31 g/L, KH_2PO_4, 12.54 g/L K_2HPO_4) supplemented with 0.1 mg/mL ampicillin and 0.034 mg/L chloramphenicol, for 12 h at 37 °C under constant shaking. This starting culture was used to inoculate 950 mL of TB medium, which was maintained at 37 °C under constant shaking until reaching 0.6–0.8 optical density at 600 nm (OD_{600}). Expression of recombinant protein was induced by adding 1 mM IPTG and incubating cultures for 5 h at 37 °C, under constant shaking. The recombinant protein was isolated by centrifugation at 10,000 × *g* for 30 min at 4 °C and subsequent solubilization of the cellular pellet in high concentration of denaturing agents (6 M Urea, 10 mM Tris–Cl, 100 mM NaH_2PO_4 and 15 mM imidazole), followed by cell disruption using a Branson digital sonifier (Branson, Los Angeles, USA). The recombinant protein was purified from the supernatant by affinity chromatography using Ni^{2+}-NTA agarose (Qiagen, CA), as recommended by the manufacturer.

Elution fractions obtained from affinity chromatography were separated by SDS-PAGE in 10% gels and electrotransferred to nitrocellulose membrane. Membranes were blocked under constant shaking for 1 h with 5% skimmed milk in phosphate buffered saline (PBS) containing 0.05% Tween and then washed thrice with PBS–Tween 0.05%. Membranes were incubated with a 1:4500 dilution of anti-polyhistidine monoclonal antibody coupled to peroxidase (SIGMA) for 2 h at room temperature under constant shaking, then washed thrice with PBS–Tween 0.05% and finally developed using the kit "VIP Peroxidase substrate" (Vector Laboratories, Burlingame, CAN), according to the manufacturer's instructions. Fractions containing the recombinant protein were pooled and refolded by thorough dialysis against PBS, pH 7.4, and then concentrated by ultrafiltration. Protein content was determined by the bicinchoninic acid (BCA) method, using bovine serum albumin (BSA) as standard [18].

2.2. Immunization assays

Immunization assays were carried out in splenectomized *Aotus* spp. adult monkeys from the Colombian Amazon basin, which were maintained and taken care under the constant supervision of a primatologist and according to the conditions stipulated by the Colombian Ministry of Health (law 84/1989). A total of 33 monkeys previously determined to be seronegative for *Plasmodium* spp. by immunofluorescence antibody test (IFAT) were randomly distributed into different immunization groups. Three groups, each containing seven monkeys, were inoculated with 50 μg of rPvMSP10, formulated in Freund's complete adjuvant, Montanide ISA720 and aluminum hydroxide, respectively. Three groups of four monkeys each was inoculated on the same day with PBS emulsified in one of the three aforementioned adjuvants to be used as controls. Sera were collected from peripheral blood samples on days 0, 20, 40 and 60 for immunological assays.

2.3. Challenge and parasitemia assessment

All monkeys were intravenously inoculated 20 days after receiving the third immunization dose with 2.5×10^6 *P. vivax* VCG-1 strain asexual blood-stage parasites. Starting on day 4 post-challenge, monkeys were followed up daily for parasitemia development by microscopic examination of Giemsa- and Acridine orange-stained blood smears. Monkeys were immediately treated with chloroquine (15 mg/day) for 2 days whenever parasitemias were ≥6% or on day 17 after concluding the assay. All monkeys were kept in quarantine until assuring they were in good health conditions and total clearance of parasites from their blood was confirmed. Monkeys were then released back into their natural habitat.

2.4. Antigenicity and immunogenicity assays

2.4.1. Western blotting

rPvMSP10 was separated by SDS-PAGE and electrotransferred for Western blot analysis, as described elsewhere [19]. Briefly, membrane strips were individually incubated with monkeys' pre-immune and immune sera as well as with serum samples from *P. vivax* patients and healthy individuals who voluntarily agreed to participate in this study, using a 1:4000 dilution of peroxidase-coupled goat anti-*Aotus* IgG or anti-human IgG as secondary antibody, respectively.

2.4.2. Enzyme-linked immunosorbent assay (ELISA)

rPvMSP10 recognition and antibody titers were determined by ELISA according to previously reported procedures [20]. In brief, 96-well plates coated with 1 μg rPvMSP10 were incubated with a 1:100

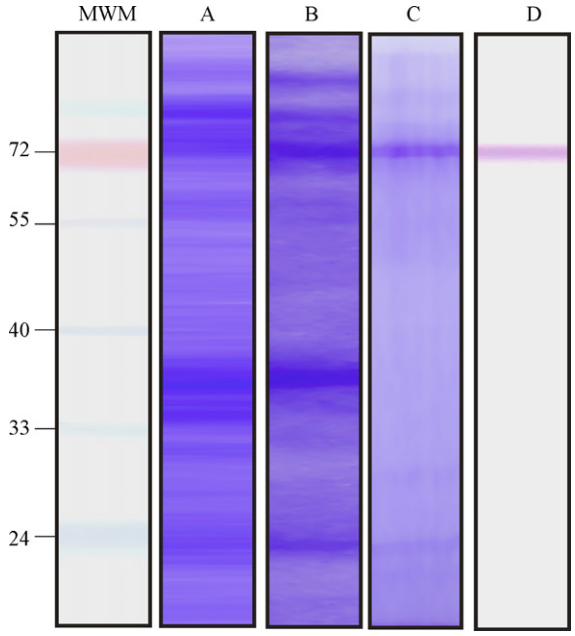

Fig. 1. SDS-PAGE and Western blot analysis of rPvMSP10 expression and purification. (A) Lysate supernatants from uninduced cells. (B) Lysate from induced cells. (MWM) Molecular weight marker. (C) Coomassie staining of affinity purified rPvMSP10 after SDS-PAGE. (D) Western blot analysis of purified rPvMSP10.

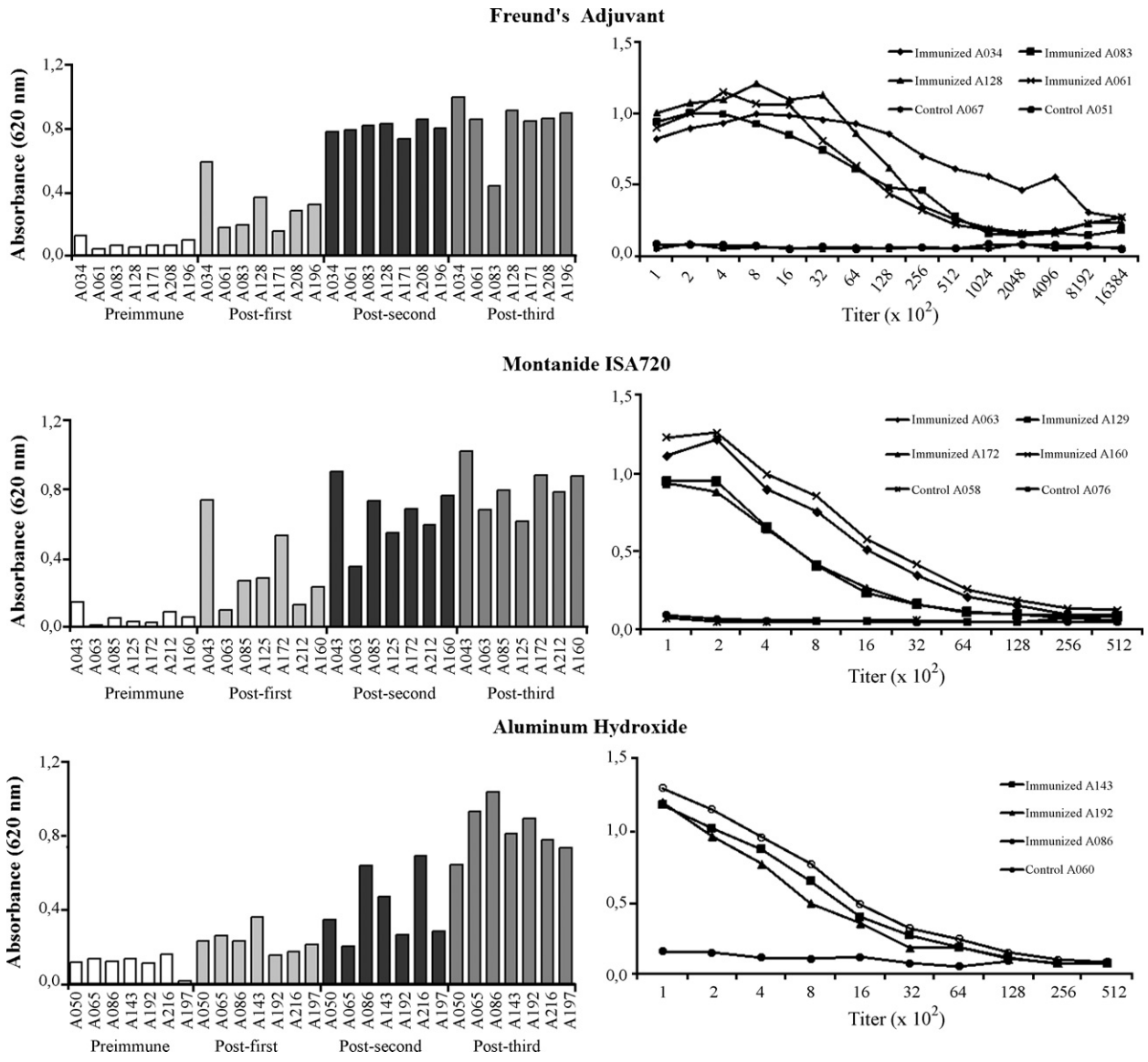

Fig. 2. Humoral immune response induced by rPvMSP10 in *A. nancymaae* monkeys with three different adjuvants, as assessed by ELISA. Left-hand panels: Recognition of rPvMSP10 by sera from immunized monkeys. Right-hand panels: Antibody titers estimated by successive 1:1 dilutions of monkey sera, until reaching the blank's absorbance ±2 S.D.

dilution of serum samples from *P. vivax*-infected individuals or immunized monkeys as primary antibody and 1:8000 anti-human IgG or 1:10,000 peroxidase-coupled anti-*Aotus* IgG as secondary antibody.

2.5. Immunofluorescence antibody test (IFAT)

P. vivax schizont-rich samples were used for IFATs. Briefly, parasites were washed thrice with PBS (pH 7.2), then centrifuged at 2500 × *g* for 4 min and suspended in 1:1 PBS–FBS (fetal bovine serum). This solution was loaded on eight-well multi-test slides and let to air dry at room temperature for 24 h. Each well was blocked with 5% BSA in PBS for 30 min and incubated with hyperimmune sera as primary antibody diluted 1:40 in blocking solution and then with fluorescein isothiocyanate (FITC)-conjugated anti-*Aotus* IgG diluted 1:100 in blocking solution as secondary antibody. Slides were then examined by fluorescence microscopy in an Olympus BX551 equipment.

2.6. Statistical analysis

Differences in antibody production between pre-immune and post-third immunization sera within each group and between the different monkey groups were evaluated using non-parametric tests (Kruskal–Wallis rank sum test and Wilcoxon signed rank test). Additionally, a non-paired test was used to determine differences in rPvMSP10 recognition by sera of *P. vivax* malaria patients and non-infected individuals.

3. Results

3.1. rPvMSP10 expression in E. coli

The MSP10 protein of *P. vivax* was expressed as a recombinant in the *E. coli* RG strain. Since rPvMSP10 formed inclusion bodies, it was necessary to use high concentrations of denaturing agents to assure solubilization. rPvMSP10 expression was observed after induction with IPTG (Fig. 1A and B), detecting a single band of

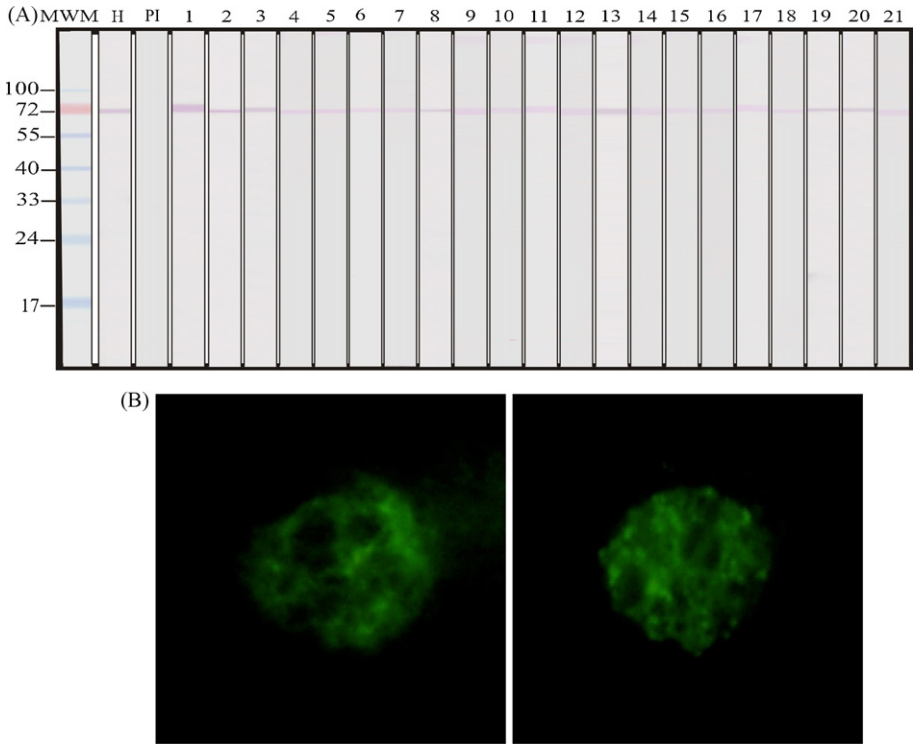

Fig. 3. (A) Western blot analysis of antibodies induced by immunization with rPvMSP10 in *Aotus* monkeys. MWM: molecular weight marker; H: positive control; PI: pre-immune sera. (1–21) Sera from immunized monkeys. (B) Cellular recognition of native PvMSP10 by sera from immunized monkeys, as assessed by indirect immunofluorescence.

~72 kDa with anti-His antibodies after purification by affinity chromatography under denaturing conditions (Fig. 1C and D). rPvMSP10 was refolded by thorough dialysis against PBS, then concentrated by ultrafiltration and quantified by the BCA method [18].

3.2. Assessing the immune response induced by rPvMSP10

3.2.1. Humoral immune response induced in Aotus monkeys

The immunogenicity of rPvMSP10 in the three monkey groups immunized with different adjuvants was evaluated by ELISA and Western blot. A significant increase in antibody production was observed after the second dose when rPvMSP10 was formulated in Freund's adjuvant and Montanide ISA720, which remained stable in post-third immunization sera (p-value 0.0078) (Fig. 2A and B). On the contrary, rPvMSP10 immunization with aluminum hydroxide induced an increase in antibody production only after the third dose (Fig. 2C) (p-value 0.0078). There were no statistically significant differences in post-third dose antibody levels between the three adjuvants (p-value 0.7404).

Specific anti-rPvMSP10 antibody titers were obtained by successive dilutions of immunized monkeys' sera, finding a 1:102,400 titer when rPvMSP10 was formulated in Freund, 1:12,800 in Montanide ISA720 and 1:6400 in aluminum hydroxide (Fig. 2, right-hand panels). There were no detectable levels of anti-rPvMSP10 antibodies in control groups being immunized with PBS in the same three adjuvants. Additionally, a single band of ~72 kDa was detected by Western blot when the serum from each of the monkeys was incubated with rPvMSP10 electrotransferred to nitrocellulose membrane (Fig. 3A).

3.2.2. Cellular recognition of native MSP10 by sera of immunized monkeys

IFATs showed that anti-rPvMSP10 antibodies recognized the protein as natively expressed by the parasite in *P. vivax* VCG-1 late schizonts, as indicated by the grape-like pattern typical of merozoite surface proteins observed under fluorescence microscopy (Fig. 3B).

3.3. rPvMSP10 recognition by sera from P. vivax malaria-infected individuals

Sera from nine patients presenting episodes of *P. vivax* malaria and living in endemic regions of Colombia (Los llanos, Norte de Santander, Guaviare and Tumaco) were used to evaluate the antigenicity of rPvMSP10 by ELISA and Western Blot. All serum samples showed high reactivity against rPvMSP10 (A_{620} 0.81 ± 0.20), compared to samples from healthy individuals (A_{620} 0.25 ± 0.01) (p-value 0.02448). Moreover, all sera reacted with a band of ~72 kDa in the Western blot analysis, indicating that the recombinant protein was recognized by antibodies naturally induced by *P. vivax* malaria infection (data not shown).

3.4. Immunization, challenge and assessment of protective efficacy

Fig. 4 shows the parasitemia profile of each monkey group receiving rPvMSP10 in three different adjuvants after being challenged with the *P. vivax* VCG-1 strain. Between days 6 and 8, monkeys in the three immunization groups developed parasitemia levels ranging between 1% and 3%, which were not different from those detected in control monkeys, therefore indicating that rPvMSP10 antibodies were not protective. However, two monkeys (A152 and A063) maintained low parasitemia levels, compared to controls.

4. Discussion

Merozoite surface proteins comprise one of the best characterized protein families in *P. falciparum*, which has been considered as promising vaccine candidates due to their accessibility to inter-

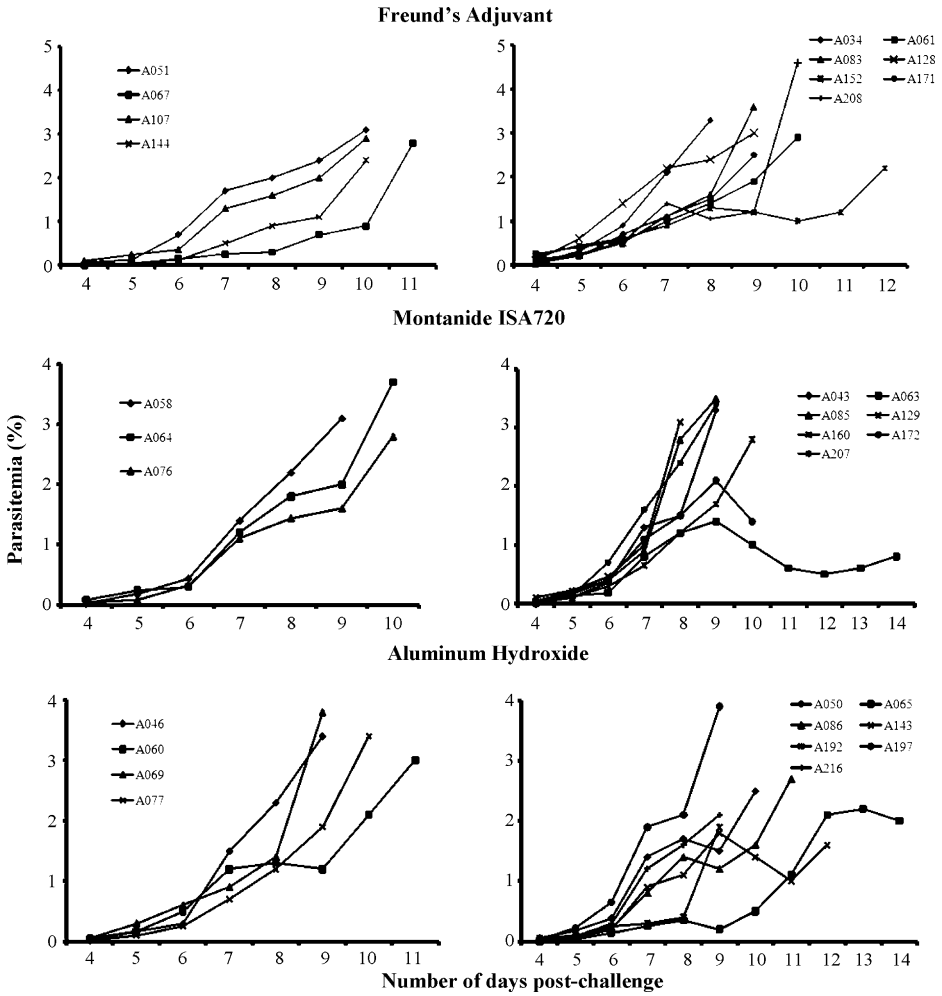

Fig. 4. Parasitemia levels developed in *Aotus* monkeys immunized with rPvMSP10 formulated with Freund's adjuvant, Montanide ISA720 or aluminum hydroxide, after being challenged with the *P. vivax* VCG-1 strain. Left-hand panel: control monkeys inoculated with PBS in one of the adjuvants being assessed. Right-hand panel: immunized monkeys.

action with host's immune system molecules [15]. Of the 11 MSPs identified to date, MSP1, MSP4, MSP5, MSP8 and MSP10 are known to contain one or two EGF-like domains, which have been proposed to act as ligands during merozoite invasion to RBCs and are highly antigenic, immunogenic and functionally conserved among the different *Plasmodium* species [21,22].

Among the MSPs, PfMSP10 is a polypeptide comprising 524 residues that contains two EGF-like domains as well as a GPI-anchoring site and has also been suggested to undergo post-translational modification same as other MSPs [23]. PfMSP10 is a target of antibodies from naturally infected individuals and it has been shown that three peptides derived from this protein bind with high affinity and specificity to surface proteins on human RBCs inhibiting *in vitro* invasion of merozoites by high percentages [24]. Based on this evidence, a PfMSP10 homologous was identified in *P. vivax* by screening the genome of *P. falciparum*. Such homologue, named PvMSP10, showed a high degree of identity with the EGF-like domains of PfMSP10, contained also a hydrophobic GPI-anchoring region and was proteolytically cleaved into two fragments of ~75 and ~58 kDa [14]. Additionally, recent data from the *P. vivax* transcriptome shows that the protein is expressed between 35 and 43 h of the intraerythrocytic cycle, same as other protein implicated in RBC invasion [5].

In this study, PvMSP10 was expressed in *E. coli*, purified by affinity chromatography under denaturing conditions and refolded by thorough dialysis. The SDS-PAGE and Western blot analysis with anti-polyhistidine antibodies detected a band with an apparent molecular weight of 72 kDa, which is higher than the one estimated based on the primary sequence of PvMSP10 (52 kDa). This phenomenon has been repeatedly observed in numerous malaria antigens, including the MSP10 from *P. falciparum*, but the origin of such molecular weight increase remains unknown [23,25].

In general, vaccination with rPvMSP10 formulated in three different adjuvants: Freund's adjuvant, Montanide ISA720 and aluminum hydroxide induced strong immunogenicity (Fig. 2). Higher antibody titers were induced when rPvMSP10 was formulated with Freund's adjuvant, which combines the immunomodulatory properties of *Mycobacterium tuberculosis* with good short term depots, but its use in humans is not allowed due to its severe side effects [26]. Montanide ISA720 and aluminum hydroxide, both of which are approved for human use, also induced high antibody titers, being this consistent with several immunogenicity studies with malaria antigens showing higher antibody production with Montanide ISA720 than with aluminum hydroxide. Additionally, more than two boosters were required when aluminum hydroxide was used as adjuvant to produce comparable antibody levels to the ones induced by Montanide ISA720 and Freund's adjuvant, which is possibly related to the moderate depot effect of aluminum hydroxide. Interestingly, the results of this study show

higher antibody production with aluminum hydroxide in comparison with immunization trials carried out in monkeys or in phase I trials where poor immune responses have been found when using this adjuvant in combination with several malarial antigens [27,28].

On the other hand, antibodies raised by rPvMSP10 in all three formulations recognized PvMSP10 natively expressed on late schizonts, as indicated by the grape-like fluorescence pattern (Fig. 3B), which confirms the proper refolding of the rPvMSP10 used in immunization assays. Moreover, sera from humans infected with *P. vivax* also recognized rPvMSP10, indicating that both forms of the protein share common epitopes targeted by antibodies induced during a natural infection.

Despite the induction of high antibody titers by immunization with the complete rPvMSP10, such antibodies showed no protective efficacy in *Aotus* monkeys against experimental challenge with *P. vivax* VCG-1 strain, independently from the adjuvant being used (Fig. 4). Such lack of correlation between immunogenic and protective properties has been observed in other antigens such as PvRBP1 and PfMSP1 [20,29].

Previous immunogenicity studies in the *Aotus* animal model conducted by our group have shown that conserved synthetic peptides derived from *P. falciparum* proteins such as EBA-175, MSP1, RESA and ABRA are neither immunogenic nor protection-inducing. Such lack of protection has been associated with the poor or imperfect adjustment of the peptide inside the groove of class II major histocompatibility (MHC) molecules, which prevents formation of a stable MHC–peptide–TCR complex needed to induce an effective immune response. This imperfect fit of conserved and functionally relevant peptides that should be, in theory, the ideal vaccine candidates, constitutes another effective evasion mechanism of the immune response by *Plasmodium* parasites [30]. Nevertheless, it has been also found that specific amino acid replacements made to such peptides (performed according to physicochemical parameters) induce shifts in their tridimensional structure, improving their immunogenicity and protection-inducing ability [30]. Based on such approach, preliminary ligand–receptor interaction studies have been conducted with *P. vivax* proteins, such as MSP-1, RBP1 and DBP, leading to the identification of peptides binding with high activity to reticulocytes [31–33]. It would be therefore also relevant to identify binding regions of MSP10 to host cells and assess the immunogenicity of structurally modified MSP10 binding regions, given that RBC invasion is not effectively blocked nor reduced by antibodies induced by immunization with the unmodified rMSP10.

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