Plasmodium vivax thrombospondin related adhesion protein: immunogenicity and protective efficacy in rodents and *Aotus* monkeys

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The thrombospondin related adhesion protein (TRAP) is a malaria pre-erythrocytic antigen currently pursued as malaria vaccine candidate to Plasmodium falciparum. *In this study, a long synthetic peptide (LSP) representing a* P. vivax *TRAP fragment involved in hepatocyte invasion was formulated in both Freund and Montanide ISA 720 adjutants and administered by IM and subcutaneous routes to BALB/c mice and* Aotus *monkeys. We measured specific humoral immune responses in both animal species and performed a sporozoite challenge in* Aotus *monkeys to assess the protective efficacy of the vaccine. After immunization both mice and* Aotus *seroconverted as shown by ELISA, and the specific anti-peptide antibodies cross reacted with the parasite in IFAT assays. Only two out of six immunized animals became infected after* P. vivax *sporozoite challenge as compared with four out of six animals from the control group. These results suggest that this TRAP fragment has protective potential against* P. vivax *malaria and deserves further studies as vaccine candidate.*

Key words: *Plasmodium vivax* - malaria vaccine - thrombospondin-related adhesion protein

Thrombospondin related adhesion protein (TRAP) or sporozoite surface protein 2 (SSP2/TRAP) is an antigen from the pre-erythrocyte stage of the malaria parasite *Plasmodium*. The protein has been found present in the sporozoite micronemes and appears to be released to the parasite surface immediately before the invasion process. It is a transmembrane protein that belongs to the TRAP/Micronemal protein 2 (TRAP/MIC2) family required for sporozoite gliding motility and together with the circumsporozoite protein (CS) it has been found to be essential to the process of malaria sporozoite infection to the hepatocyte (Muller 1993, Sinnis & Sim 1997). This protein highly conserved among *Plasmodium* species contains, towards the N-terminus of the protein in *P. vivax,* a conserved fragment (WTP**CSVTCG**VGVRVRRR) denominated region II-plus (Sinnis & Sim 1997) located at positions (307-324) which bind to sulfated glycoconjugates (Templeton & Kaslow 1997). Like the malaria CS protein, SSP2/TRAP binds to the heparan sulfate molecules of the basolateral domain of hepatocytes during the initial contact between hepatocytes and the invading sporozoites (Frevert et al. 1993, Yuda & Ishino 2004).

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Several studies using functional in vitro assays and animals models such as mice and non-human primates have demonstrated that TRAP can block the hepatocytes invasion by sporozoites and therefore confers partial protection against *P. yoelii, P. cynomolgi,* and *P. falciparum* infection (Hoffman et al. 1996).

In addition, it has been observed that both, humans vaccinated with *P. falciparum* irradiated sporozoites or individuals naturally exposed to malaria induce $CD8^+$ T-cell-dependent cytolytic activity mediated by a HLA-restricted epitope derived from SSP2/TRAP, indicating its potential as vaccine candidate (Aidoo et al. 1995, Wizel et al 1995). However, there are no published studies on the immunogenicity or protective efficacy of the *P. vivax* TRAP. Here we describe a pilot immunogenicity study conducted in BALB/c mice using the *P. vivax* TRAP derived long synthetic peptide (LSP) which contains the motif for sporozoites and hepatocytes interaction, followed by a pre-clinical study in *Aotus* monkeys to assess its protective potential.

MATERIALS AND METHODS

Peptide - We synthesized a peptide corresponding to the amino-acid sequence 209-256 of Salvador I strain *P. vivax* TRAP (PvTRAP) located towards the N-terminus of the protein, which contains the region II motif involved in binding parasites in hepatocytes (Templeton & Kaslow 1997) (see Fig. 1). This peptide was synthesized under good laboratory practices (GLP) conditions using F-moc chemistry. The peptide contains 6 cysteine residues which were oxidized according to Corradin et al. (2004) and then it was purified by high-performance liquid chromatography (HPLC) to purity higher than 80% (Sigma Genosys, St Louis, Mo).

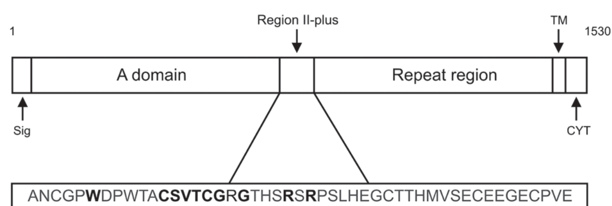

Fig. 1: schematic representation of *Plasmodium vivax* TRAP protein showing the localization of the synthetic peptide used in the present study. Sig: signal sequence; TM: transmembrane sequence; CYT: cytoplasmic tail. Bold characters represent the cell adhesive motif

Animals - BALB/c mice and *Aotus lemurinus griseimembra* monkeys from the animal facility of the Malaria Vaccine and Drug Development Center (MVDC) Cali, Colombia, were used. *Aotus* monkeys were adult male and non-pregnant females, malaria naïve, with body weight above 800 g. Animals were overseen by a veterinarian and were handled under US National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals. The animal testing protocol was submitted to and approved by the Animal Ethics Committee of the Universidad del Valle.

Immunization schedules - For the pilot study, eight BALB/c mice were separated in two groups of four animals each: the experimental group was immunized on days 0, 30, and 60 by subcutaneous (SC) route with 30 µg of PvTRAP formulated in Freund's adjuvants at a 1:1 proportion. The control group was immunized with phosphate buffer saline (PBS) formulated in the same adjuvants following similar immunization schedule. The first vaccination dose was formulated in complete Freund adjuvant (CFA), whereas the other two doses were formulated in incomplete Freund adjuvant. Monkeys were randomly assigned to two groups of six animals per group. The experimental group received IM injections of 100 µg PvTRAP peptide formulated in Montanide ISA 720 (SEPPIC, Paris, France) in a proportion of 3:7 antigen:adjuvant as recommended by the manufacturer. Injections were given on days 0, 30, and 90 and a fourth vaccination dose with the PvTRAP peptide formulated in CFA was given on day 150 to determine the potential boosting effect. This latter vaccination was administered subcutaneously, with the LSP formulated in a 1:1 proportion in a mixture (50:50) of CFA and incomplete Freund's adjuvants. The six animals in the control group followed the same immunization schedule as the experimental group but with adjuvant emulsified in PBS without peptides.

Antibody determination - Enzyme-linked immunosorbent assay (ELISA) was done to determine antibody titers to the LSP, using sera from experimental and control animals, collected on the day of each immunization as described previously (Arévalo-Herrera et al. 1998). Briefly, ELISA well plates (Nunc-Immuno Plate, Maxisorp, Roskilde, Denmark) were coated overnight with the LSP (1 µg/ml) in PBS pH 7.2 at 4ºC. After blocking with 5% BSA in PBS, serial dilutions of test serum were added and incubated for 1 h at room temperature. Then, antibodies were detected using phosphatase-conjugated anti-mouse IgG (Sigma Chemical Co., St Louis, MO) in case of rodent and with phosphatase-conjugated anti-human IgG for monkeys (Sigma), and the plates were developed using an enzymatic reaction with para-nitrophenyl phosphate substrate. The results were expressed as the reciprocal of the endpoint of the last dilution at which OD_{450} for control serum (pre-immunization serum) was greater than the average (OD_{450}) plus three standard deviations (SD).

Native protein recognition - We performed indirect immunofluorescence assays (IFAT) to assess the recognition of the *P. vivax* native TRAP by specific anti-peptide antibodies, IFAT multi-well slides were prepared using 10 µl of a parasite suspension containing 2×10^3 *P. vivax* sporozoites obtained from experimentally infected *Anopheles albimanus* as described before (Herrera et al. 1997). Slides were stored frozen at –70°C until use. For IFAT the slides were thawed and dried at room temperature and then incubated with double serial dilutions of antibodies from experimental and control mice and monkeys for 1 h. Three washes were done, and reaction was developed with fluorescein-conjugated goat antimouse IgG (H+L) in the case of rodent sera, and with fluorescein-conjugated goat antihuman IgG (H+L) (Jackson Immunoresearch Laboratories, Inc., Baltimore, MD) diluted at 1:100 for the monkey studies. The slides were examined by epifluorescence microscope. Antibody titers were expressed as the positive end point dilution that showed specific fluorescence of the sporozoite surface.

Aotus IFN-γ ELISPOT - To determine IFN-γ production by specific mononuclear cells induced by synthetic peptide, ELISPOT was carried out using a human IFN-γ kit (Mabtech, Stockholm, Sweden) as described elsewhere (Herrera et al. 1997). Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood of experimental and control monkeys on days 0, 30, 90, and 150 by Ficoll-Histopaque density gradient. A total of 2×10^5 cells per well were cultured in plates (Millipore, MAHA S45, Bedford, MA, US) for 40 h at 37°C and 5% CO_2 in the presence of 10 µg/ml of PvTRAP peptide. The plates were pre-coated overnight with mouse antihuman IFN-γ monoclonal antibodies (1-DIK Mabtech) blocked with Roswell Park Memorial Institute (RPMI) containing 10% fetal calf serum for 2 h at 37 °C and 5% CO_2. Plates were washed and treated with biotinylated goat antihuman IFN-γ polyclonal antibody followed by color development using the reagents supplied in the kit. IFN-γ cell production was determined by comparison with positive controls stimulated with phytohemaglutinine (PHA) and expressed as 10^6 spot-forming units (SFU).

Protective efficacy of vaccination - We attempted to assess the efficacy of vaccination to prevent infection after challenge with viable *P. vivax* sporozoites, using prepatent period, parasitemia levels and duration of infection and anemia as markers of protection. *Aotus*

were intravenously inoculated with 2×10^4 *P. vivax* sporozoites obtained by salivary gland dissection of *A. albimanus* mosquitoes, previously fed with blood from a human patient as previously described (Jordan et al. 2005). Infection was performed on day 60 after last immunization (day 210). Giemsa stained thick and thin blood smears (WHO 1991) were done before challenge and every other day from day 15 after challenge to detect parasitemia. We used the thin smear to determine the percentage of infected red blood cells (RBC) contained in a total of 1000 RBC, whereas thick smears were used to score the number of parasites in 300 leucocytes. We defined presence of one parasite as positive and marking the end of the prepatent period. Each slide was read independently by two microscopists in a blinded manner for group and sample day. To confirm the results, all samples that were negative by thin and thick smears were tested by a nested polymerase chain reaction (PCR) on Whatman filter paper. Filter paper was soaked in 100% methanol for 45 min, then methanol was removed and DNA was precipitated from methanol with 50 µl of water and used to perform a species-specific nested PCR as previously described (Snounou et al 1993). In order to determine the potential development of anemia, all monkeys were subjected to hematocrit and hemoglobin determination every other day on the same days as parasitemia.

Statistical analysis - Protective efficacy was evaluated by comparing the incidence of infection and survival time between the experimental and control groups and differences were determined using square chi test. Incidence of infection was defined as the number of positive cases over the number of animals exposed to infection. In addition, proportion of animals without infection for each group was defined as the time from inoculation of sporozoites to the development of infection detected by PCR. Estimated proportion curves were calculated using the Kaplan Meier method (Berry et al. 1992). Differences between experimental and control groups were analyzed by a log rank test conducted at the two-sided, 0.05 level of significance.

RESULTS

Immunogenicity of PvTRAP peptide - All BALB/c mice seroconverted after the first immunization dose as determined by ELISA. Titers of anti-peptide antibodies increased steadily after the first immunization to reach titers ranging from 1 to 128 x 10^3 after the third injection (Table I). Similarly, sera of two of the four mice vaccinated with the TRAP LSP were able to recognize sporozoites in IFAT assays. Although all *Aotus* monkeys also seroconverted after the complete immunization regime, antibody levels that ranged from 1×10^2 to 1×10^4 were significantly lower than those of mice. Most animals needed at least two immunization doses of PvTRAP peptide formulated in Montanide ISA 720 adjuvant to produce detectable antibody levels, and four of the six monkeys produced a rapid decrease of specific antibody titer after the third immunization to undetectable levels by day 150 (Table II). However, all monkeys produced a rapid antibody boost (1 x 10^4) after inoculation with PvTRAP formulated in Freund´s adjuvant on day 150. At these anti-peptide titers, parasites were recognized in IFAT assays at dilutions up to 1:400 (Table II).

No difference in IFN-g production by mononuclear cells stimulated with PvTRAP peptide was observed between experimental ($15\text{-}38 \times 10^6$) and control ($14\text{-}45 \times 10^6$) groups. Cells from all animals responded to PHA stimulation ($26\text{-}465 \times 10^6$) (Table III).

Protective efficacy of PvTRAP peptide - We initially intended to measure three outcomes to assess the protective efficacy of PvTRAP peptide: prepatent period, degree of parasitemia, and duration of infection, determined by thick and thin smears after parasite challenge. However, these two tests were found to be negative in all experimental and control monkeys. We therefore used nested PCR. Two of out six monkeys in the experimental group showed parasitemia by PCR, with prepatent periods of 16 and 53 days, whereas four out of six monkeys from the control group became infected, with a prepatent period of 53 days in one monkey and 16 days in the three remaining animals.

TABLE I

Titers of anti-PvTRAP antibodies developed in BALB/c mice

	ELISA[a]				IFAT[b]			
Mouse/day	0	30	60	90	0	30	60	90
E1	0	2	16	64	0	100	400	400
E2	0	2	2	1	0	0	0	0
E3	0	2	16	16	0	100	200	400
E4	0	2	64	128	0	0	0	0

a: Elisa anti-PvTRAP antibodies titer expressed as the reciprocal of the last positive dilution $\times 10^3$; *b*: IFAT performed with air-dried *P. vivax* sporozoites. Titers represent the reciprocal of the serum end-point dilution.

TABLE II

Titers of anti-PvTRAP peptide antibodies developed in of *Aotus* monkey

	ELISA[a]						IFAT[b]
Mouse/day	0[c]	30	60	105	150	170	170
277*543	0	0	1	5	1	10	40
096*060	0	0	1	5	0	10	40
347*512	0	0	1	1	0	10	40
318*026	0	0.1	1	1	0	10	40
584*533	0	0	0.1	1	0	10	neg
119*322	0	0	1	5	0	10	neg

a: ELISA anti-PvTRAP antibodies titer expressed as the reciprocal of the last positive dilution $\times 10^3$; *b*: IFAT performed with air-dried *P. vivax* sporozoites. Titers represent the reciprocal of the serum end-point dilution; *c*: underline numbers correspond to days of immunization.

TABLE III

Production of IFN-γ by mononuclear cells from peripheral blood of *Aotus* monkeys immunized with PvTRAP peptide

Group	Monkey	0	30	90	105	0	30	90	105
		Peptide				PHA			
		Spot-forming units (millons)							
Experimental	277*543	35	67.5	5	22.5	27.5	222.5	355	335
	096*060	15	65	38	17.5	17.5	165	290	475
	347*512	30	5	0	0	5	115	465	377.5
	318*026	5	33	48	0	35	142.5	480	265
	584*533	0	10	0	10	12.5	87.5	460	555
	119*322	20	50	35	40	80	130	380	372.5
	Average	17.5	38.3	20.8	15	29.6	143.8	405.0	396.7
	SD	14	27	21	15	26.9	46.5	75.6	103.2
Control	352*009	42.5	8	8	0	12.5	192.5	382.5	340
	779*631	35	60	8	38	12.5	200	460	567.5
	306*297	2.5	28	33	28	75	175	442.5	540
	085*836	0	58	25	0	25	195	432.5	477.5
	115*890	7.5	35	20	23	5	112.5	420	317.5
	835*827	0	83	30	0	27.5	182.5	340	552.5
	Average	14.6	45.0	20.4	14.6	26.3	176.3	412.9	465.8
	SD	19.1	26.8	10.9	16.7	25	33	44	111

INF-γ production by mononuclear cells from experimental monkeys immunized with synthetic peptide formulated in Montanide ISA-720 by I.M. (0, 30, and 90) and SC booster with peptide formulated in Freund's adjutants on 150 day and control monkeys immunized at same time only whit adjuvant

Fig. 2 shows the proportion of animals without infection following inoculation in experimental and control groups after challenge with *P. vivax* sporozoites, detected by PCR. Sixty-seven percent of monkeys immunized with PvTRAP peptide survived to the end of follow-up (day 69) without becoming infected. In contrast, only 33% of the control group survived to day 69 (Chi-squared log-rank test: 6.3 1gl; p: 0.01). The two-fold difference in the incidence of infection in experimental and control groups was not statistically significant (p: 0.24). All monkeys presented normal hematocrit and blood hemoglobin concentration from day 0 to day 210, the end of observation (data not shown). No differences between groups were observed.

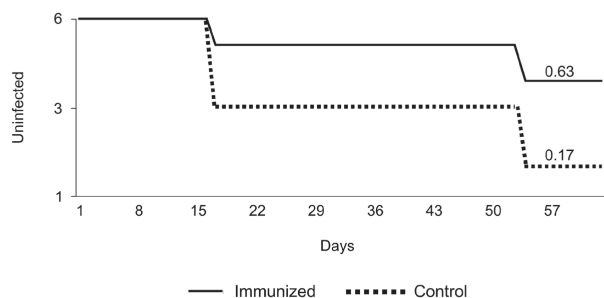

Fig. 2: proportion of animals that remain free of infection after challenge with 10^5 *Plasmodium vivax* heterologous parasites detected by PCR of *Aotus* monkeys immunized with PvTRAP peptide and control monkeys immunized with adjuvant alone.

DISCUSSION

Development of a *P. vivax* malaria vaccine that blocks sporozoite invasion to the liver cell or its further intracellular multiplication would appear ideal, since this parasite species is characterized by the development of hypnozoites which are responsible for the periodic relapses induced by this parasite species. In this respect the complete protection achieved by vaccination with *P. falciparum* and *P. vivax* irradiated sporozoites and the partial protection accomplished by vaccination of human volunteers with the *P. falciparum* RTS-S (Alonso et al. 2004) have generated sufficient evidence for the possibility to successfully vaccinate humans against malaria.

P. falciparum TRAP has been considered another potential vaccine subunit. However, a Pf-TRAP based DNA vaccine (MVA ME-TRAP) could not be protected from natural infection, although apparently the vaccine was able to reduce the number of parasites that developed in the liver (Moorthy et al. 2004). No data are available about studies conducted with *P. vivax* TRAP.

In the search for *P. vivax* malaria parasite subunits with vaccine potential, here we have focused efforts towards the N-terminal region of the *P. vivax* TRAP known to contain a binding motif for invasion of liver cells. In this study we evaluated the immunogenicity and protective efficacy of a LSP containing this RII binding domain in rodents and non-human primates. The peptide proved to be immunogenic in both animal species when formulated in Freund's adjuvant not acceptable for human use, as well as in Montanide ISA 720 currently being used in human vaccine trials. Although, mice developed a steadily increasing antibody pattern since the first immunization and those antibodies cross reacted with

the parasite in a dose dependent manner, antibodies from monkeys immunized with the peptide formulated in Montanide ISA 720 were very low. However, significant boosting was observed when the peptide formulated in Freund adjuvant was administered as well as cross reactivity with the parasite. These results indirectly confirm the good immunogenicity of the peptide provided a potent adjuvant is used, and indicate that Montanide ISA 720 is likely to be an inadequate adjuvant for inducing high levels of specific antibodies for the TRAP peptide tested; in fact several vaccine doses were required in monkeys to induce detectable antibody responses. These results are in contrast with a number of trials performed by our group where all formulations containing Montanide ISA 720 induced a rapid antibody response that usually lasted for about one year (Arévalo-Herrera et al. 2005a,b, Herrera et al. 2005). Furthermore, T cell responses appear to be the most efficient mechanisms of protection against malaria pre-erythrocytic forms; however in this study the production of IFN-γ specifically in response to stimulation with PvTRAP peptide was not demonstrated most probably due to ineffective stimulation of $CD4^+$ and/or $CD8^+$ T-cells by the vaccine formulation used. Alternatively, it is possible that production of IFN-γ levels in *Aotus* by specific lymphocytes in response to PvTRAP peptide is dose-dependent and could not be detected with the immunization dose or the dose for in vitro stimulation used in this study.

Although the protection attained in this first study, was not statistically significant, the immunogenicity achieved in terms of anti-peptide antibody response that cross reacted with the parasites, together with the trend of parasitemia in control and vaccinated animals, represents an important preliminary result that deserves further studies with more potent adjuvants and an optimized sporozoite challenge system. Here we used a wild parasite strain that had not been previously adapted to grow in monkey; this may explain the difference with previous studies from our group (Jordan et al. 2005).

Finally, most probably multiple vaccine subunits would be required to achieve acceptable malaria protection. It has been proposed that the CS and TRAP, the two *P. vivax* pre-erythrocytic vaccine candidates described so far, might function synergistically by blocking sporozoite invasion, as they appear to have different antibodies binding specificities that may function at different stages of hepatocytes invasion (Sinnis & Sim 1997). In conclusion, we believe that these preliminary results provide valuable bases for further studies to assess TRAP alone and in combination with other *P. vivax* subunit vaccine (i.e. CS, MSP-1 or others) in preclinical studies.

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