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Gamma interferon levels and antibody production induced by two PvMSP-1 recombinant polypeptides are associated with protective immunity against *P. vivax* in *Aotus* monkeys

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Abstract

Effector mechanisms responsible for providing protective immunity against *Plasmodium vivax* (*Pv*) infection were examined in *Aotus* monkeys vaccinated with two *Pv* Merozoite Surface Protein-1 (*Pv*MSP-1) recombinant polypeptides that had previously been shown to protect vaccines against parasite challenge. Vaccine efficacy was reproducible in this trial, showing that one out of the five monkeys immunised with the recombinant protein mixture was partially protected while three others controlled parasitaemia. Antibodies reactive to the parasite's native proteins, the recombinant polypeptides and peptides spanning both recombinant fragments were detected in most vaccinees. Despite substantial Peripheral Blood Mononuclear Cell (PBMC) antigen-specific cellular proliferation not being detected, high r*Pv*MSP-1_{20} specific gamma interferon (IFN-γ) production was found in the three animals that controlled parasitaemia. Altogether the results suggest that antibody titres and antigen-specific IFN-γ production mediate protective immunity against *P. vivax*.

Keywords: *Plasmodium vivax*; MSP-1 33 kDa fragment; Immunisation; Immune response

1. Introduction

Plasmodium vivax causes an estimated 80 million cases of malaria annually in South and Central America, India, South-east Asia and Oceania. Furthermore, the increasingly high drug resistance currently displayed by this species [1] reinforces the need for developing an effective vaccine for controlling infections caused by this parasite. *Aotus* monkeys displaying high immune system homology with that of humans [2–5] have become a useful animal model when selecting antigens [6–8] and evaluating the immune response directed towards malaria vaccine candidates [9,10].

MSP-1 from several *Plasmodium* species has been extensively tested in *Aotus* and mice as one of the antigens explored as a anti-malarial vaccine candidate [11–14]; different recombinant segments derived from the *Pv* MSP-1 protein are currently being evaluated [15–18]. The *Pv* VCG-1 strain adapted to splenectomised *Aotus nancymaae* monkeys [19] has allowed us to evaluate the protective efficacy of two recombinant polypeptides (r*Pv*MSP-1_{14} and r*Pv*MSP-1_{20}) encoding almost the entire *Pv*MSP-1 33 kDa cleavage fragment. These sequences fulfil a good anti-*P. vivax* vaccine candidate's criteria: the encoded sequences are highly conserved throughout natural isolates, they harbour peptide sequences exhibiting reticulocyte binding activity and the polypeptides are widely recognised by *Pv* infected patients' sera [20]. Cellular im-

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mune mechanisms have not been evaluated even though this protein's fragments have conferred protection and elicited antibodies in vaccinees when administered with Freund's adjuvant [11]. Since characterising the immune response against r*Pv*MSP-1_{14} and r*Pv*MSP-1_{20} vaccine candidates will shed light on the immune mechanisms behind protective immunity against malaria and might contribute towards developing effective anti-malarial vaccines, this work evaluates antigen-specific antibody production, PBMCs proliferation and IFN-γ production generated when immunising *Aotus* monkeys with r*Pv*MSP-1_{14} and r*Pv*MSP-1_{20} recombinant segments and their relationship with protective efficacy.

2. Materials and methods

2.1. Immunisation, boosting and challenge

Ten splenectomised *Aotus nancymaae* monkeys from the Colombian Amazon region having no evidence of previous *Plasmodium* infection as assessed by indirect immunofluorescence assays (IIFA) [11] were thus used. Animal care and handling followed international animal care guidelines [21]. Five animals were immunised with r*Pv*MSP-1_{14} and r*Pv*MSP-1_{20} recombinant polypeptides expressed in *Escherichia coli* as previously reported [20]. A 50 μg antigen/dose emulsified in 300 μl (v/v) Freund's complete adjuvant was injected (first dose), followed by two similar doses in incomplete adjuvant 30 days apart. Control group consisted of five monkeys immunised with adjuvant plus PBS. Animals were bled prior to beginning immunisation (PI), then 20 days after each antigen dose (I_{20}, II_{20} and III_{20}) and 60 days post-parasite challenge (PC) for obtaining PBMCs and plasma. Animals were challenged twenty days after the third immunisation and parasitaemia was monitored daily as described in [11]. All infected monkeys were treated on day 16 with quinine and clyndamicin (30 mg/day/5 day), sulphadoxine-pyrimethamine 260 mg and primaquine 750 μg (unique/dose), or before whenever parasitaemia levels reached ≥6% or when an animal's life became compromised because of severe anaemia, according to conditions reported in previous studies which showed that prolonging the time after 16 days did not lead to changes in parasitaemia [11,19].

2.2. Humoral immune response

Antibody production elicited by vaccination with recombinant polypeptides was measured by two approaches. Antibody titres against native protein in serial PI, I_{20}, II_{20} and III_{20} plasma dilutions (starting at 1:40) were determined by IIFA using *P. vivax* air-dried slides having high trophozoite and schizont content.

ELISA was used for measuring the reactivity of antibodies elicited against r*Pv*MSP-1_{14}, r*Pv*MSP-1_{20} to ten peptides representing these polypeptides' entire sequence for mapping peptide epitopes within the two polypeptides (Fig. 3). Peptides 1735 (^{1339} EILVPAGISDYDVVYLKPLA), 1736 (^{1359}GMYKTIKKQLENHVNAFNTN), 1737 (^{1379}ITDMLDSRLKKRNYFLEVLN), 1738 (^{1399}SDLNPFKYSPSGEYIIKDPY), 1739 (^{1419}KLLDLEKKKKLLGSYKYIGA), 1745 (^{1539}KVINNCQLEKKEAEITVKKL), 1746 (^{1559}QDYNKMDEKLEEYKKSEKKN), 1747 (^{1579}EVKSSGLLEKLMKSKLIKEN), 1748 (^{1599}ESKEILSQLLNVQTQLLTMS) and 1749 (^{1619}SEHTCIDTNVPDNAACYRYL) were synthesised by SPPS t-boc [22] (the first number represents start amino-acid position according to the *Pv* Belem strain sequence). One μg antigen (recombinant proteins or peptides) per well, suspended in 100 μl 25% PBS-DMSO, was added to a 96-well ELISA plate and incubated overnight. After being incubated with 100 μl of a 1:50 plasma-dilution for 3 h at 37 °C, wells were washed and covered with 100 μl purified peroxidase-labelled goat anti-*Aotus* IgG (diluted 1:8,000) for 1 h at 37 °C. The reaction was developed with 100 μl peroxidase substrate (KPL) and absorbance measured at 620 nm.

2.3. Cellular immune response

1×10^5 PBMCs obtained by gradient centrifugation in Ficoll-Hypaque (1.077 density) (Lymphoprep, Nycomed Pharma AS, Oslo, Norway) were cultured in triplicate in 100 μl complete medium (RPMI-1640 supplemented with 5×10^{-5} M 2-mercaptoethanol and 10% autologous plasma) in 96-well plates and stimulated with 10 μg/ml of each recombinant protein or PHA (Difco, Michigan, USA) for examining cellular immune response in vaccinees. After 72 h culturing at 37 °C and 5% CO_2, supernatants were harvested (for IFN-γ quantification by ELISA) and cultures pulsed with 1 μCi [^{3}H]-thymidine (Amersham-Pharmacia, Buckinghamshire, UK) 16 h prior to harvesting. Thymidine incorporation was measured by liquid scintillation in a beta counter. The results are expressed as stimulation index (SI), determined as mean counts per minute (cpm) incorporated in a protein-stimulated culture/mean cpm in control cultures incubated without antigen. An SI equal to or higher than 2 was considered positive.

IFN-γ quantified in culture supernatants for assessing cellular response status in vaccinees by using a commercial kit for human IFN-γ (OptEIA, Pharmingen, San Diego, CA). Statistically significant differences between immunised and control groups' IFN-γ levels were evaluated by using parametric *t*-test or non-parametric Kruskall–Wallis analysis.

3. Results and discussion

It was found that while all monkeys in the control group developed around 6% parasitaemia (days 9–13), 4/5 immunised monkeys controlled infection; monkey 23756 was partially protected (1.72% parasitaemia on day-9 clearing parasite-infection afterwards) and monkeys 23291, 23640 and 23676 controlled infection (3.5, 4.06 and 3.27 %

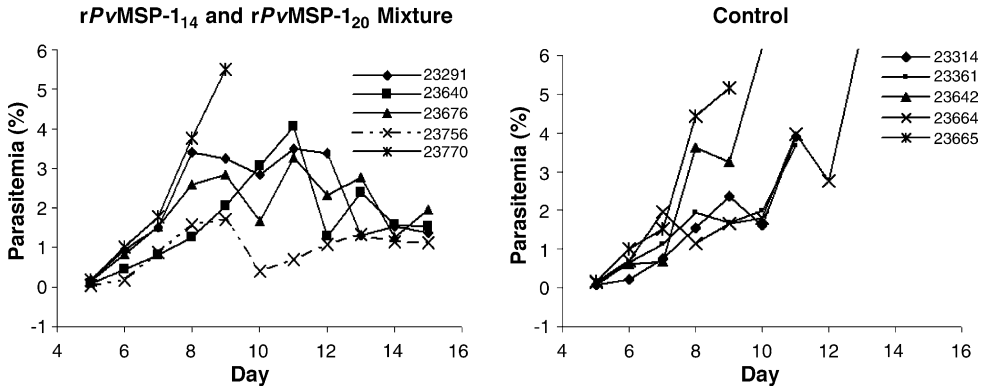

Fig. 1. Parasitaemia curves from the immunised splenectomised *Aotus nancymaae* monkeys; vaccinees were immunised with r*Pv*MSP-1_{14} and r*Pv*MSP-1_{20} while controls were immunised with just PBS. Parasitaemia was evaluated daily following challenge up to day 16 by acridine orange staining. Parasitaemia was reported as being parasitised red blood cell percentage.

maximum parasitaemia on day 11, respectively). Only monkey 23770 displayed a similar behaviour to that of the control group (Fig. 1). Hence, the previously described protective efficacy [11] was reproducible in this study.

All immunised monkeys (except for 23770) displayed antibodies against *Pv* able to recognise the native protein in the III_{20} sample. The highest antibody titres (1:160) in the III_{20} were found in monkey 23756 that completely controlled parasitaemia (Fig. 2). Neither PI plasmas nor those from control animals had detectable antibodies (Table 1). These results suggest that the recombinant polypeptides elicited PvMSP-1-specific antibodies protecting *Aotus* vaccinees against *Pv* infection.

Aotus IgGs recognising each recombinant protein were found in all immunised monkeys, the amount for PvMSP-1_{20} being higher than that for PvMSP-1_{14}. Plasma IgGs have also been detected 60 days after parasite challenge in the three monkeys where this was examined (Fig. 3).

Interestingly, priming IgG production against recombinant polypeptides after parasite challenge was detected in the 2 control animals evaluated (see A_{620} PC plasmas in Fig. 3). Since monkey 23756 displayed the lowest parasitaemia and exhibited the highest antibody titres by IIFA, we decided to examine IgG fine specificity elicited against peptide epitopes within the recombinant polypeptides. This analysis showed specific IgG levels for peptides 1737, 1738 and 1749 in the III_{20} sample. The following can be concluded since IgGs specific for 1739 and 1747 peptides were only detected in immunised animals being challenged with the parasite: there was boosting of peptide-specific IgG production as a consequence of parasite challenge; anamnestic anti-peptide antibodies persisted 60 days after challenge; and this recognition was immunisation-dependant and not derived just from parasite exposure. Regarding antibody response measured by ELISA, it is worth noting that higher absorbance was detected when recombinant proteins were used as antigens, probably corresponding to IgGs directed towards multiple epitopes being detected (Fig. 3).

Proliferation results concerning PBMCs (III_{20} bleeding) being stimulated in vitro with recombinant polypeptides indicated that whereas mitogen response was observed in most animals' cell cultures, no significant stimulation indexes in response to the polypeptides (Table 2, panel A) were found in vaccinees. Null response to mitogen observed in monkeys 23291 and 23640 might be explained by previously reported intrinsic differences in cellular proliferation to mitogen among individual monkeys [23].

Vigorous IFN-γ production against the r*Pv*MSP-1_{20} protein was found in monkeys 23291, 23640 and 23676 (Table 2,

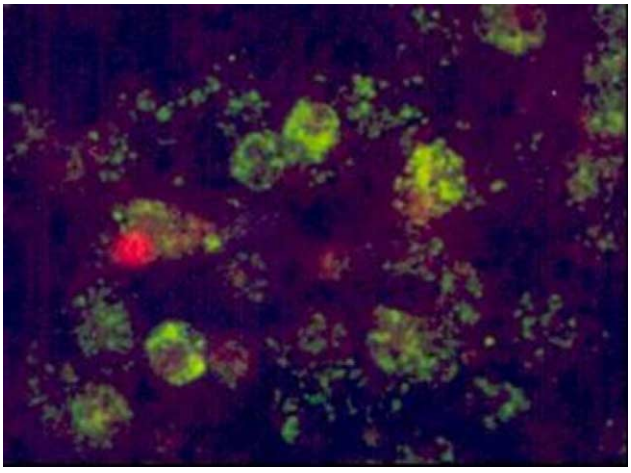

Fig. 2. IIFA. Indirect Immunofluorescence microscopy of *P. vivax* mature schizonts using 23756 monkey serum III_{20} sample in a 1:160 dilution.

Table 1
Antibody titres induced by immunising splenectomised *Aotus nancymaae* monkeys with the protein mixture

Inoculated antigen	*P. vivax* (trophozoites-schizonts)			
	PI	I_{20}	II_{20}	III_{20}
r*Pv*MSP-1_{20}/r*Pv*MSP-1_{14}	0/5	2/5	4/5	4/5
Adjuvant/PBS only	0/5	0/5	0/5	0/5

The data reflects the number of monkeys having antibody titres recognising *P. vivax* vs. the total number of each group.

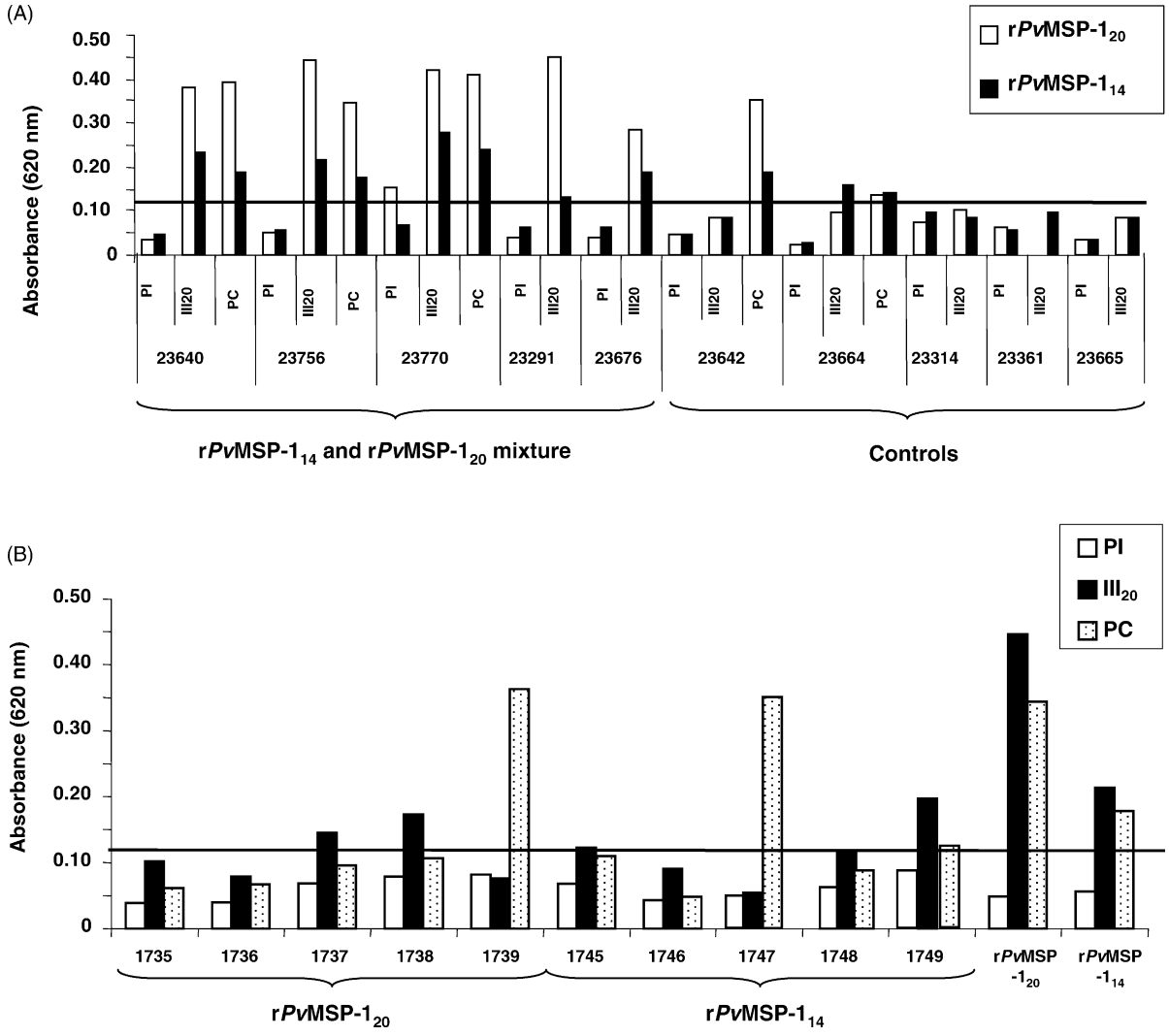

Fig. 3. ELISA (A) Recognition of r*Pv*MSP-1_{20} and r*Pv*MSP-1_{14} recombinant polypeptides. Immunised monkeys' plasma samples: PI (prior to immunisations being begun), III_{20} (20 days after the third immunisation) and PC (60 days post-challenge). (B) Monkey 23756 PI, III_{20} and PC sample recognition of each peptide spanning the whole of the recombinant proteins' sequences. Absorbance can be seen on the vertical axis; the horizontal line indicates average absorbance from PI plasma plus three standard deviations.

Table 2
PBMC III_{20} lymphoproliferation and IFN-γ production after r*Pv*MSP-1_{14} and r*Pv*MSP-1_{20} recombinant protein stimulation

In vitro stimulus	r*Pv*MSP-1_{14} and r*Pv*MSP-1_{20} mixture					Controls				
	23291	23640	23676	23756	23770	23314	23261	23642	23664	23665
S.I. III_{20}										
r*Pv*MSP-1_{20}	1	1	1	1	0	0	1	1	1	1
r*Pv*MSP-1_{14}	1	***2***	0	1	0	0	1	1	1	1
PHA	1	1	**13**	**6**	**11**	**32**	**9**	**11**	**16**	**3**
IFN-γ (pg/ml) III_{20}										
r*Pv*MSP-1_{20}	***198***	***211***	***249***	58	37	49	21	26	37	25
r*Pv*MSP-1_{14}	29	56	45	50	50	68	23	36	32	37
PHA	**36**	**33**	**281**	**334**	**237**	**217**	**237**	**440**	**222**	**207**

SI: stimulation index.

panel B). A statistically significant difference between immunised and control groups' IFN-γ levels was found by using parametric (*t*-test) or non-parametric analysis (Kruskall–Wallis); *P* values corresponded to 0.0252 and 0.0072, respectively. These results agree with previous studies demonstrating association between antigen-specific IFN-γ production and reduced pathology in humans [24] and those in *Aotus* monkeys suggesting that high IFN-γ levels may contribute towards controlling *P. falciparum* parasitaemia in animals immunised with MSP-1 [25].

Even though IFN-γ production was observed in most partially protected monkeys, monkey 23756 (partially protected) did not produce IFN-γ but displayed the highest antibody titres against the native protein as determined by IIFA. The importance of having antibodies directed towards the native antigen is highlighted by the results observed in monkey 23770 (not protected) which did not show antibodies by IIFA, even though antibody levels against the denatured protein as determined by ELISA were found.

This study has been aimed at elucidating the immune system mechanisms triggered when splenectomised *Aotus nancymaae* monkeys are immunised with r*Pv*MSP-1_{14} and r*Pv*MSP-1_{20} proteins; their relationship to protection has also been discussed. Monkeys immunised with the two recombinant proteins were able to control infection in experimental challenge with *P. vivax* due to specific antibodies as well as antigen-specific IFN-γ production by PBMCs. Compared to our previous work [11], increasing the immunisation schedule by one dose favoured antibody production and establishing a direct correlation between antibody production and protection. These results, as well as previously reported antigenicity, immunogenicity, low variability, reticulocyte-binding and protection studies concerning r*Pv*MSP-1_{14} and r*Pv*MSP-1_{20} recombinant proteins [26], support these polypeptides' being included in a multi-stage multi-component anti-*P. vivax* malaria vaccine.

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