Immunization of *Saimiri sciureus* Monkeys with *Plasmodium falciparum* Merozoite Surface Protein-3 and Glutamate-Rich Protein Suggests that Protection is Related to Antibody Levels

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Abstract

The immunogenicity and protective efficacy of various antigen-adjuvant formulations derived either from the merozoite-surface protein-3 (MSP-3) or the glutamate-rich protein (GLURP) of *Plasmodium falciparum* were evaluated in *Saimiri sciureus* monkeys. These proteins were selected for immunogenicity studies based primarily on their capacity of inducing an antibody-dependent cellular inhibition effect on parasite growth. Some of the *S. sciureus* monkeys immunized with MSP-$3_{212-380}$-AS02 or GLURP$_{27-500}$-alum were able to fully or partially control parasitaemia upon an experimental *P. falciparum* [Falciparum Uganda Palo Alto (FUP-SP) strain] blood-stage infection, and this protection was related to the prechallenge antibody titres induced. The data are indicative that MSP-3 and GLURP can induce protective immunity against an experimental *P. falciparum* infection using adjuvants that are acceptable for human use and this should trigger further studies with those new antigens.

Introduction

The role of antibodies in the immunity against the blood stages of *Plasmodium falciparum* malaria has been well established by passive transfer experiments performed by Cohen and McGregor [1] and later by Bouharoun-Tayoun and coworkers [2]. In the latter, immunoglobulins (Igs) obtained from malaria immune African individuals caused striking decrease in parasitaemia when passively transferred to nonimmune Thai patients. The same Ig preparations active *in vivo* were unable to directly inhibit parasite growth *in vitro* but showed a strong inhibitory effect when allowed to cooperate with human monocytes in an antibody-dependent cellular inhibition (ADCI) fashion. The ADCI assay has thus been considered a good *in vitro* correlate of antimalaria immunity acting *in vivo* [3] and has since been used to screen for potential malaria vaccine candidate antigens. Antibodies against merozoite-surface protein-3 (MSP-3) [4] and glutamate-rich protein (GLURP) [5] strongly inhibit *P. falciparum* growth in ADCI assays. Evaluation of the fine specificity of affinity purified anti-MSP-3 and anti-GLURP antibodies revealed that those directed against the MSP-3b and P3 epitopes mediated the strongest ADCI effects [4, 6]. Immuno-epidemiological studies have demonstrated that high levels of cytophilic MSP-3- and GLURP-specific antibodies are significantly associated with protection against *P. falciparum* malaria [4, 7, 8]. The B-cell epitopes that are targeted by these human antibodies are highly conserved among isolates from different geographical origins [9, 10], suggesting that they are functionally constrained and not subjected to selection for variation. Such characteristics support the further evaluation of MSP-3 and GLURP as malaria vaccine candidate antigens. In the present work, we have proceeded to a preliminary preclinical evaluation of several constructs derived from MSP-3 and GLURP in combination with different adjuvants in the New World primate *Saimiri sciureus*, which is together with Aotus [11, 12], one of the WHO-recommended

primate models for malaria research [13, 14]. The first goal was to assess in comparative manner the immunogenicity of various antigen-adjuvant formulations, and the second goal was to gather a preliminary indication of the possible protection induced upon *falciparum* challenge.

Materials and methods

Antigens and formulations. Seven different antigen-adjuvant combinations were tested in this work (six with MSP-3 and one with GLURP). The recombinant protein DG210 [4], covering the central nonrepetitive region of MSP-3 (amino acids 193–257), was used in combination with incomplete Freund's adjuvant (IFA) (Sigma Chemical Co., St. Louis, MO, USA). The small MSP-3b peptide (aa 211–237), representing a major T- and B-cell epitope within the DG210 protein, was presented as an eight-branch multiple antigen peptide (MAP) construct without adjuvant or as the monomer in combination with IFA. A recombinant protein covering the nonpolymorphic carboxy-terminal region of the MSP-3 protein (aa 212–380) was used in combination with AS02 (formerly SBAS2 – Glaxo SmithKline, Rixensart, Belgium), Montanide ISA720 (Seppic, Paris, France) or Ribi adjuvants. The recombinant protein R0, covering the nonrepetitive conserved amino terminal region of GLURP (aa 27–500) was supplemented with $Al(OH)_3$ (alum) (Superfos Biosector, Vaerloese, Denmark).

The nonrepetitive $glurp_{79-1500}$ 5′-region was amplified with the primers 5′-AAA *AGA TCT* ACA AGT GAG AAT AGA AAT AAA C (nucleotides 79–100) and 5′-AAA *AGA TCT* TGC TTC ATG CTC GCT TTT TTC CGA T (nucleotides 1500–1476) [counting from A in the ATG start codon of M59706], and the $msp3_{628-1140}$ 3′-region was amplified with the primers 5′-AAA *AGA TCT* AAA GCA AAA GAA GCT TCT AGT TAT GAT TAT (nucleotides 628–657) and 5′-AAA *AGA TCT* TTA ATG ATT TTT AAA ATA TTT GGA TAA (nucleotides 1143–1117) [counting from A in the ATG start codon of L07944] using DNA from Falciparum Vietnam Oak-Knoll (FVO) strain as template [10]. All amplifications were carried out for 30 cycles with denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min and extension at 72 °C for 1 min using *Taq* polymerase. Polymerase chain reaction fragments were digested with *Bgl*II (underlined) and cloned into the expression plasmid pMCT3 [15] cut with *Bgl*II, yielding plasmids pMST83 and pMST72, respectively. The $GLURP_{27-500}$ and $MSP\text{-}3_{212-380}$ regions are each fused to a stretch of six his-residues allowing purification by metal ion affinity chromatography on a Ni^{2+} column, as described previously [15].

Animals. In the immunization experiments 1 and 2 (see below), 15 wild-captured adult *Saimiri sciureus* monkeys trapped in the Marajo island, at the mouth of the Amazon River, and kept at the National Primate Center/Funasa, Belém, Brazil, were used. Upon arrival in the primate centre, nearly 100 monkeys were quarantined and a check up was performed, with evaluation of standard haematological and biochemical parameters and search for parasitic infections, including malaria (thick and thin blood-film examination and immunofluorescence for *P. brasilianum* and *P. falciparum*). Many individuals presented intestinal and/or blood (microfilaria) parasites; all were negative for *Plasmodium* species. All monkeys received antiparasitic treatment (Ivermectin 1 mg/kg) during quarantine and yearly thereafter, and stools were periodically examined (each 3 months). After quarantine, animals stayed at least 6 months in the colony before proceeding to experimentation, and at that time and thereafter, no intestinal or blood parasites were found in the 15 animals selected for experimentation. Seven individuals randomly selected from the colony were karyotyped (Dr Julio Cesar Pieczarka, Department of Genetics, Federal University of Para, Brazil). All presented $2n = 44$ chromosomes, with seven acrocentric pairs (karyotype 14–7). Given the morphotype, karyotype and geographical origin, the individuals were classified as *Saimiri sciureus sciureus* [16, 17]. Nine male (weighing 835–920 g) and six female (weighing 530–600 g) individuals were distributed in five groups of immunization and one control group (one female in each group). All animals were adults, but precise determination of age was not possible. In the immunization experiment 3, six captive-born adult *Saimiri sciureus sciureus* monkeys were used, obtained from the colony established at the Department of Primatology/CECAL/Fiocruz, Rio de Janeiro, Brazil. All individuals were male, weighing 720–860 g and aging 3–8 years old at the beginning of the experiment. In Belém and Rio de Janeiro, animals were splenectomized at least 2 months before first immunization injection. Splenectomy is necessary to allow the development of reproducible parasitaemia in *Saimiri* infected with the FUP-SP strain of *P. falciparum* (see below). The use of these animals was made in compliance with the institutional policies, and the Fiocruz Ethical Committee for Animal Experimentation approved the described protocols.

Immunization protocols. Protocols were designed to gather a preliminary assessment of: 1) safety and immunogenicity of the formulations; 2) lifespan of antibodies and induction of immunological memory and 3) protective efficacy upon experimental *P. falciparum* challenge infection. Given the second aspect, a long follow-up with intermittent booster injections was performed (Fig. 1). In all cases, a final volume of 500 µl containing 100 µg of each antigen thoroughly mixed with the respective adjuvant was administered subcutaneously in four different points in the shaved back of the animals. Nonhuman primates are a scarce resource for research work; hence we decided to perform a preliminary assessment of several antigen-adjuvant formulations in small number of *Saimiri*

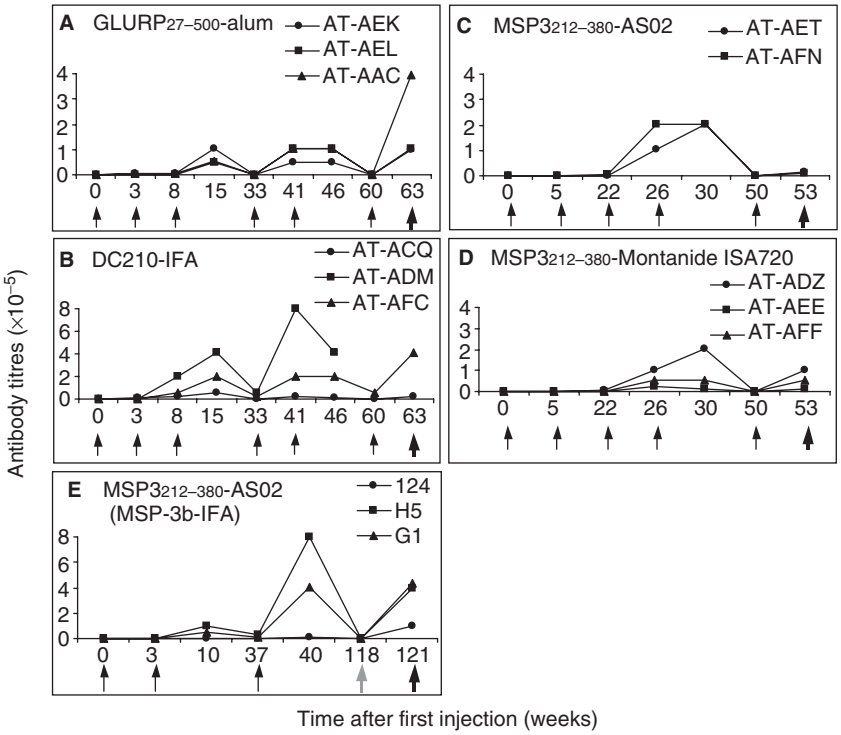

Figure 1 Follow-up of antibody titres (ELISA) during immunization of *Saimiri sciureus* monkeys with different antigen-adjuvant formulations. (A–D) Experiments 1 and 2 (thin arrows: immunization injections; thick arrows: challenge infection). (E) Experiment 3 (thin arrows: immunization with merozoite-surface protein (MSP)-3b-incomplete Freund's adjuvant (IFA); gray arrow: immunization with MSP-$3_{212-380}$-AS02; thick arrow: challenge infection). Code names of each monkey are shown on the legend on the top right side. *Y*-axis is presented with different scale in (B) and (E).

so as to select the most promising ones for larger future studies. Three separate immunization experiments were performed. *Experiment 1*: six monkeys received either DG210-IFA (three monkeys) or GLURP$_{27-500}$-alum (three monkeys). *Experiment 2*: seven monkeys received MSP-$3_{212-380}$ in combination with AS02 (two monkeys), Montanide ISA720 (three monkeys) or Ribi (two monkeys). The animals immunized with Ribi adjuvant were not challenged because this formulation was found to be ineffective in stimulating an antibody response against MSP-3. Ten of 11 remaining monkeys (one from the DG210-IFA group died) from experiments 1 and 2 were challenged at the same time, 3 weeks after a final boost injection (see below). *Experiment 3*: three monkeys received three injections of MAP-MSP-3b. As in the case of MSP-$3_{212-380}$-Ribi, MAP-MSP-3b was found to be ineffective in stimulating an antibody response against MSP-3, and these monkeys received, 5 months later, the first of three injections of MSP-3b-IFA, and 18 months after the third injection, they received one injection of MSP-$3_{212-380}$-AS02 and were challenged 4 weeks later (Fig. 1E). The reason for switching to this formulation was that its efficacy had been shown in the challenge experiment 1 (see *Results*). Moreover, AS02 adjuvant has recently undergone clinical trials and is expected to be permitted for use in humans. Just before and 3–4 weeks after each immunization injection, haematological parameters were evaluated, monkeys were weighed, and the sites of injection were examined to search for local adverse reactions.

Challenge infections. The FUP-SP strain of *P. falciparum* (a kind gift of Dr Thierry Fandeur, Institut Pasteur of French Guiana – IPG) was used in the challenge experiments. This strain was adapted to splenectomized *Saimiri sciureus* in the late 1970s [18]. After nearly 100 serial passages, this strain has become highly virulent for naïve splenectomized animals, which develop fast rising parasitaemia consistently requiring drug treatment [13, 19–21], usually administered when parasitaemia reaches 20%. Lethal infection can be induced with the inoculation of less than 100 parasitized red blood cells (pRBCs) (T. Fandeur, personal communication). This model has been well established and was found to be highly reproducible in the host/parasite combination used in the present work (splenectomized *Saimiri sciureus* karyotype 14–7/*P. falciparum* FUP-SP).

In the immunization experiments 1 and 2, the 10 immunized *Saimiri* monkeys presenting antibodies against both the immunogen and the parasite were challenged with 5000 *P. falciparum* (FUP-SP strain) pRBC, with the predominance of ring and young trophozoite stages, obtained from a donor monkey. Two nonimmunized naïve *Saimiri* monkeys were used as controls. The relatively low inoculum was chosen in view of the reported virulence of the FUP-SP strain. In the immunization experiment 3, the three monkeys receiving MSP-$3_{212-380}$-AS02 and three naïve control monkeys were challenged with 50,000 *P. falciparum* pRBC of the same strain (seven passages from the original aliquot). Despite the fact that in the challenge experiment 1, the low inoculum caused, as expected, fast rising parasitaemia in the two control monkeys (see *Results*), the inoculum size in the challenge experiment 2 was increased one log to ascertain that the

apparent protection observed in the experiment 1 could be achieved using a higher inoculum. Parasites were given intravenously and parasitaemia was daily followed-up by the examination of Giemsa-stained thick and thin smears of blood obtained from the footpad. As masking procedure for determining parasitaemia, animals were assigned a different number each day, according to a random order at which they were taken from the cages for preparing the blood films, of which the microscopist was not aware. Rectal temperature was daily evaluated and haematocrit checked every 4 days. Monkeys were treated with chloroquine (three daily doses of 10 mg/kg) whenever parasitaemia reached 20% (first challenge) or 10% (second challenge) and/or when the haematocrit went below 25%. The reduced threshold of parasitaemia for treatment in the second challenge experiment was decided because in the first one some monkeys presented major falls in haematocrit.

Immunological assays. All immuno assays were performed prior to conducting challenge.

Enzyme-linked immunosorbent assay. Ninety-six-well plates (Maxisorp, Nunc, Denmark) were coated with 1 μg/ml (MSP-$3_{212-380}$ or $GLURP_{27-500}$ proteins) or 2 μg/ml (MSP-3b peptide) of the test antigen, 100 μl/well in carbonate-bicarbonate buffer (pH 9.6) overnight at 4 °C. Uncoated sites were blocked with 200 μl of phosphate-buffered saline (PBS)/0.05% Tween 20 (PBS-T) containing 3% nonfat milk for 1 h at room temperature (RT) and the wells washed three times with PBS-T. Serial dilutions of each serum sample were prepared in each plate with PBS-T/1% nonfat milk and incubated for 1 h at RT. Preimmunization serum samples and sera from nonimmunized animals were used as controls. Plates were washed three times with PBS-T and a rabbit anti-*Saimiri* IgG (1 : 8000) was added and the plates incubated for 1 h at RT. After washing, a goat antirabbit IgG conjugated to peroxidase (Sigma A-9169, St. Louis, MO, USA) (1 : 5000) was added and incubated for 1 h at RT. After washing, 100 μl of a solution of orthophenylediamine (OPD) in citrate-phosphate buffer (10 mg/25 ml plus 10 μl H_2O_2) (pH 5.0) was added to each well and incubated for 30 min at RT in the dark, and then 50 μl/well of H_2SO_4 2N was used to stop the reaction. Plates were read at 492 nm in a spectrophotometer (Spectramax 250, Molecular Devices, Sunnyvale, USA). Cut-off values were defined as the mean optical density (OD) of control wells plus three standard deviations (blank subtracted).

ELISA for GLURP epitope mapping. Plates were coated with 2.5 μg/ml of Streptavidin (Sigma) in citrate-phosphate buffer (pH 5.0) overnight at 4 °C. After washing with 0.5 M NaCl-PBS/T20 0.1%, the biotin-conjugated peptides P3, P4, P5, P8, P9, P10, P11 and S3, covering known B-cell epitopes within the $GLURP_{27-500}$ region [6], were added at 1 μg/ml in 0.37 M NaCl-PBS/T20 0.1%, buffer, for 1 h at RT. After washing, serum samples diluted at 1 : 200 in 0.7 M NaCl-PBS/T20 0.1% were added for 1 h at RT. Plates were washed and a rabbit anti-*Saimiri* IgG (1 : 8000) was added and the plates incubated for 1 h at RT. After washing, a goat antirabbit IgG conjugated to peroxidase (Sigma A-9169) (1 : 5000) was added and incubated for 1 h at RT. Plates were washed and development was performed as in the previous item. Results were expressed as the OD ratio between each test sample and the cut-off values (mean OD + three standard deviations of nonimmunized, noninfected *Saimiri* serum samples) for each peptide. The reactivity of each serum against $GLURP_{27-500}$ was assessed in parallel at a 1 : 200 dilution.

Immunofluorescence antibody test. Immunofluorescence antibody test (IFAT) was performed using *P. falciparum* (FCR-3 strain, schizont stage) obtained from *in vitro* cultures synchronized with metrizamide. Serum samples were diluted in PBS, added to the slides and incubated at 37 °C for 40 min. After extensive washings in PBS, a goat antihuman IgG conjugated to fluorescein (Sigma) diluted at 1 : 250 in PBS/Evans Blue was added and the slides incubated at 37 °C for 40 min and then washed, dried, mounted with a coverslip using buffered glycerin solution and read in a fluorescence microscope (Zeiss). Endpoint titres were determined on the wells giving fluorescence over the background of preimmunization serum samples.

Statistical analysis. To analyse the relationship between antibody titres and outcome of infection, an Independent-Samples *T*-test was used. Due to the low number of animals and the variance in antibody titres, the Levene's Test for equality of variances was applied previous to analysis. To check the hypothesis that antibody titres above a given threshold would be protective, findings were dichotomized and the Fisher Exact's Test was applied to compare samples above or below median antibody titre values (percentile 50).

Results

Immunogenicity and memory response

Among the seven antigen-adjuvant formulations tested, five were shown to be immunogenic, eliciting antibody responses (Fig. 1A–E). In all cases, the overall profile of antibody induction was similar. Titres increased mainly after the second or the third injection. There was a decrease in titres several weeks after each booster injection, but an extra dose was able to promptly stimulate a memory response, even when given several months after the previous injection. Antibody levels were variable among different groups and within each group. In addition, despite the induction of antigen-specific antibody responses reaching titres as high as 400,000 or 800,000 in enzyme-linked immunosorbent assays (ELISAs), the recognition of the native protein on the parasite as seen in IFAT was markedly lower, titres being up to 400 or 800 (Table 1). Two formulations tested (MAP-MSP-3b and

Table 1 Individual antibody titres obtained after immunization of *Saimiri sciureus* with MSP-3 or GLURP-derived formulations and just before challenge infection with *Plasmodium falciparum*, and their relationship with outcome of infection, peak parasitaemia and prepatent period

Group	*Saimiri* code	Prechallenge antibody titre ELISA	Prechallenge antibody titre IFAT	Outcome of infection (day)	Peak parasitaemia (day)	Prepatent period (days)
GLURP$_{27-500}$-alum	AT-AAC	409,600	3200	Clearance (31)	3.3% (22)	11
	AT-AEK	102,400	400	THP (14)	>20%	07
	AT-AEL	102,400	1600	THP (18)	>20%	11
DG210-IFA	AT-ACQ	12,800	400	TSA (25)	16% (19)	12
	AT-AFC	409,600	1600	TSA (22)	4% (22)	09
MSP-3$_{212-380}$-AS02	AT-AET	12,800	50	THP (28)	>20% (28)	18
	AT-AFN	12,800	400	No infection	0%	–
MSP-3$_{212-380}$-Montanide ISA720	AT-ADZ	102,400	400	TSA (25)	19% (24)	18
	AT-AEE	25,600	200	THP (21)	>20%	14
	AT-AFF	51,200	400	TSA (22)	16% (22)	11
Nonimmunized controls	AT-AGA	negative	negative	THP (16)	>20%	10
	AT-AGG	negative	negative	THP (21)	>20%	12
MSP-3$_{212-380}$-AS02 (MSP-3b-IFA)	124	102,400	50	TSA (27)	6.7% (22)	10
	G1	409,600	800	Clearance (30)	4.5% (22)	10
	H5	409,600	800	Clearance (30)	2.4% (22)	09
Nonimmunized controls	41	negative	negative	THP (15)	>10%	07
	H13	negative	negative	TSA (25)	7.2 (23)	08
	H17	negative	negative	THP (18)	>10%	07

MSP-3, merozoite-surface protein-3; GLURP, glutamate-rich protein; ELISA, enzyme-linked immunosorbent assay; IFA, incomplete Freund's adjuvant; IFAT, Immunofluorescence antibody test; THP, treated for high parasitaemia; TSA, treated because of the development of severe anaemia.

MSP-3$_{212-380}$-Ribi) failed to induce specific antibodies in *Saimiri*, but the same antigens were shown to be immunogenic when prepared in combination with other adjuvants: MSP-3b-IFA, MSP-3$_{212-380}$-AS02, or MSP-3$_{212-380}$-Montanide ISA720.

Epitope mapping of GLURP$_{27-500}$-immunized Saimiri sera

Prechallenge serum samples from two of the three GLURP$_{27-500}$-immunized *Saimiri* showed a strong reactivity with the peptide P11 and/or peptide P5 (Table 2); serum of the third *Saimiri* – the one with highest anti-GLURP$_{27-500}$ and antiparasite antibody titres – showed a broader spectrum of reactivity, recognizing most of the peptides, but also with a stronger reactivity with P11. A pool of sera from *Saimiri* poly-infected with *P. falciparum* [pooled immune serum (PIS)] also showed a broader spectrum of reactivity, yet with stronger reactivity with P5 and P11.

Table 2 GLURP$_{27-500}$ epitope mapping: reactivity in ELISA of serum samples (diluted at 1 : 200) from GLURP$_{27-500}$-immunized (AT-AEK, AT-AEL and AT-AAC) or immune (PIS) *Saimiri sciureus* against peptides covering the GLURP$_{27-500}$ protein

Sample	Reactivity against P3	P4	P5	P8	P9	P10	P11	S3	GLURP$_{27-500}$
AT-AEK	–	–	10.4	–	–	–	2.1	1.7	40.3
AT-AEL	–	–	–	–	–	–	13.3	–	46.8
AT-AAC	1.7	1.8	2.3	1.5	1.3	1.8	8.1	1.4	42.0
PIS	1.4	–	8.2	4.7	–	–	8.9	–	44.3

Results are expressed as the optical density ratio between each test sample and the cut-off values for each peptide. Reactivity against GLURP$_{27-500}$ is shown as reference (although direct comparison is not fair because of the difference in the ELISA protocols). GLURP, glutamate-rich protein; ELISA, enzyme-linked immunosorbent assay; PIS, pooled immune serum.

Safety

All monkeys that received the IFA (with DG210 or MSP-3b) developed local inflammatory reaction at the injection site, with swelling and in some cases limited areas of skin necrosis. Reactions were observed mainly after the second injection, and increased in size and severity with additional doses. Size of lesions varied considerably, the largest recorded being 2.1 × 1.4 cm. One of three *Saimiri* receiving MSP-3$_{212-380}$-Montanide ISA720 developed a similar but less severe reaction, the area affected being smaller without necrosis, which was observed only after the final prechallenge booster dose. Local reactions were not observed in the monkeys immunized with the GLURP$_{27-500}$-alum, MSP-3$_{212-380}$-AS02 or MSP-3$_{212-380}$-Ribi. Following immunizations, no major changes in weight or haematological parameters were observed.

Challenge infections

In the challenge experiments, three different outcomes were defined: 1) clearance of infection; 2) treatment for severe anaemia (while parasitaemia below the threshold for treatment) and 3) treatment for hyperparasitaemia.

A summary of the antibody titres and outcome of infection are summarized in Table 1.

The monkeys belonging to the immunization experiments 1 and 2 and two naïve nonimmunized control monkeys were challenged with 5000 *P. falciparum* pRBC (FUP strain). As shown in the Fig. 2(A), the two control monkeys developed detectable parasitaemia from day 12, which grew exponentially and led to decide on drug treatment on day 16 and 21.

Of the three monkeys immunized with GLURP$_{27-500}$-alum, two developed fast rising parasitaemia, similar to the controls, and thus had to be treated on days 14 and 18 (Fig. 2B). The third monkey developed only a low-grade parasitaemia peaking at 3.3% on day 22 and was able to eliminate the parasite by day 31.

The parasitaemia presented by the two monkeys immunized with DG210-IFA did not reach the threshold for treatment (20%); one monkey remained in the range 2–4% for several days and the other remained under 10% (Fig. 2C). However, they were not able to fully eliminate the parasites and due to low haematocrit had to be treated on day 22 or 25 after challenge.

In the MSP-3$_{212-380}$-AS02 group, one monkey completely controlled parasite growth and the other showed a delay in the appearance of parasitaemia (Fig. 2D; Table 1); however, after onset, the parasitaemia grew and reached more than 20% by day 28, requiring treatment.

Finally, in case of MSP-3$_{212-380}$-Montanide ISA720 group, parasitaemia in one out of three monkeys had a similar behaviour to nonimmunized control monkeys, and this animal was treated on day 21 of infection (Fig. 2E); the other two maintained their parasitaemia below the threshold for treatment however, as for the DG210-IFA group, had to be treated due to the development of anaemia. Hypothermia (temperature below 38.5 °C; prechallenge range: 38.5–40.5 °C) was also observed in the animals with severe anaemia (data not shown), which required special care such as transfer to incubators and blood transfusion.

Another common feature of the immune response was a fall of antibody titres occurring within the first 2 weeks after challenge, probably reflecting an initial consumption of antibodies by the parasites, and a subsequent rise in titres due to the booster effect of the infection (data not shown). In some cases, the serological follow-up was not continued because some monkeys were presenting major falls in haematocrit, and blood withdrawals had to be restricted to a minimum to avoid an interference with the experiment and to preserve the animals.

The three *Saimiri* immunized with MSP-3$_{212-380}$-AS02 and three naïve nonimmunized control monkeys were challenged with 50,000 *P. falciparum* pRBC (FUP strain). Two of three control monkeys presented a fast rise in parasitaemia and had to be treated 16 and 20 days after inoculation (Fig. 3E,F). Two of the vaccinated monkeys were able to control parasitaemia mostly below 2%, with peaks at 2.4% and 4.5% on day 22, and were able to completely eliminate the parasites by day 30 without requiring treatment (Fig. 3B,C). The third control and the third immunized monkeys developed parasitaemia higher than the two other immunized ones (with peaks of over 6% on days 23 or 22) and despite apparently

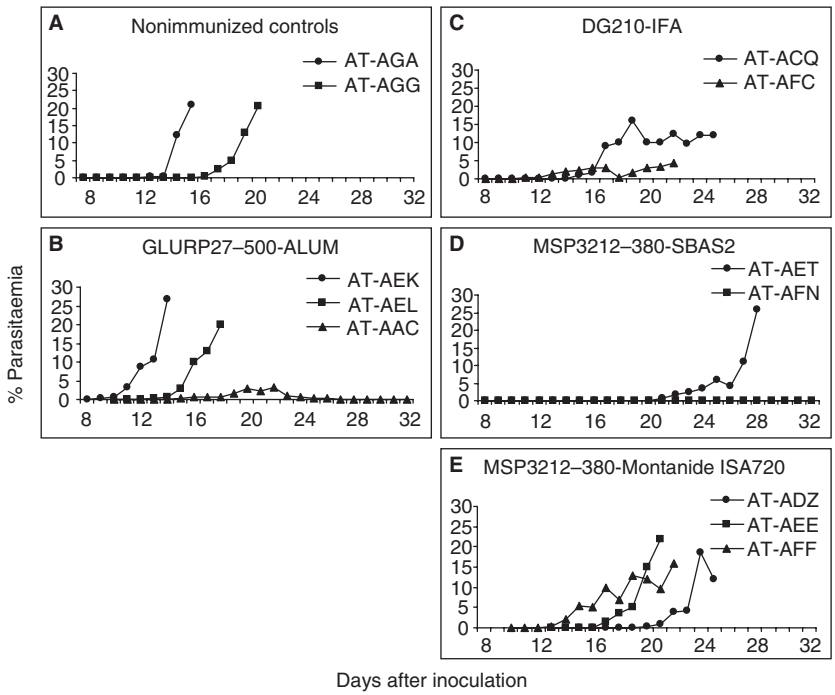

Figure 2 Challenge 1: course of parasitaemia of *Saimiri sciureus* monkeys – nonimmunized or immunized with different glutamate-rich protein (GLURP) or merozoite-surface protein (MSP)-3-derived formulations – inoculated with 5000 *Plasmodium falciparum* parasitized red blood cells (FUP strain). Code names of each monkey are shown on the legend on the top right side.

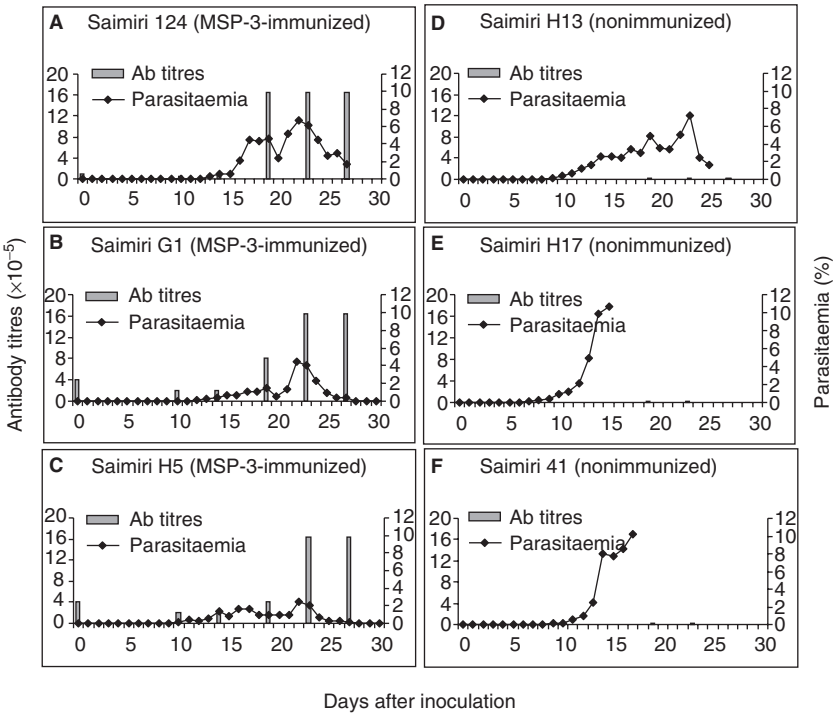

Figure 3 Challenge 2: course of parasitaemia and follow-up of anti-merozoite-surface protein (anti-MSP)-$3_{212-380}$ antibody titres (ELISA) of *Saimiri sciureus* monkeys – nonimmunized or immunized with MSP-$3_{212-380}$-AS02 – during challenge infection with 50,000 *Plasmodium falciparum* parasitized red blood cells (FUP strain).

controlling parasitaemia, had to be treated on days 25 or 27, respectively (Fig. 3A,D), because of anaemia. A clear relationship between antibody titres before and during challenge and the outcome of infection was observed, as shown in the individual graphs (Fig. 3A–F) and in the Table 1. The two fully protected monkeys had the highest anti-MSP-$3_{212-380}$ (409,600) and antiparasite (800) antibody titres, whereas the immunized monkey that developed higher parasitaemia and severe anaemia presented the lowest antibody titre (102,400 anti-MSP-$3_{212-380}$ and 50 antiparasite titres). As in the previous experiment, antibody titres decreased just after infection, and this drop correlated with an increase of parasitaemia. However, the specific response was later boosted by the infection, high antibody titres being observed after day 24. This parasite-induced increase in titres correlated with a decrease of parasitaemia finally leading to parasite elimination. In addition, prechallenge sera of the two protected monkeys, but not the immunized one that developed severe anaemia, recognized the MSP-3 polypeptide of 48 kDa in immunoblotting (data not shown). As in the previous experiment, severe anaemia was accompanied by hypothermia, and in this case, recovery from hypothermia was reflected on survival, as the nonimmunized control *Saimiri*, which showed a more striking fall in temperature (down to 36 °C), was unable to recover despite intensive care and died.

Considering all groups (Table 1), the mean antibody titres were higher in the monkeys that cleared infection (ELISA: 310 400; IFAT: 1300) than in those treated for severe anaemia (ELISA: 113 067; IFAT: 475) or treated for hyperparasitaemia (ELISA: 30 400; IFAT: 281). Similarly, monkeys developing severe anaemia were treated later (mean 24.3 days) than those having hyperparasitaemia (mean 18.8 days).

For statistical analysis of the data, given the low number of animals, individuals treated for hyperparasitaemia or for severe anaemia were grouped, although it could be assumed that a partial protection had been developed in monkeys presenting severe anaemia and low parasitaemia at the time of treatment. Due to large variance and standard deviation in antibody titres measured by ELISA, an equality of variances could not be assumed, and it was not possible to evaluate the significance of the results. However, this was possible with IFAT titres, and it was shown that monkeys that cleared infection had titres significantly higher than those nonprotected ($P = 0.041$). Moreover, parasite clearance was significantly correlated with antibody titres over 400 (percentile 50) ($P = 0.0441$).

Discussion

A malaria vaccine is expected to have a strong impact on the prevalence of this widespread disease [22], and despite the fact that it has not yet become a reality, a number of efforts is currently being undertaken to achieve this goal. In the present article, we report the immunogenicity of MSP-3 and GLURP in *S. sciureus* monkeys, and preliminary challenge data indicated that immunization, especially with the formulation MSP-$3_{212-380}$-AS02, may lead to

protection against a *P. falciparum* challenge. Anti-MSP-3 and anti-GLURP antibodies induced by infection in humans have been previously shown to be effective in controlling *P. falciparum* growth *in vitro*, acting in cooperation with monocytes (ADCI assay) [4, 5] and *in vivo* when passively transferred in the humanized severe combined immunodeficiency (SCID) mouse [23]. The data reported here bring additional indications in favour of these two vaccine candidates by showing that artificially induced anti-MSP-3 and anti-GLURP antibodies may also be effective *in vivo* when induced at high enough titres.

As a first step in our evaluation of MSP-3 and GLURP in nonhuman primates, we decided to perform a preliminary assessment of several antigen-adjuvant formulations in small number of *Saimiri* so as to select the most promising ones for larger future studies. This strategy led us to reject some antigen delivery systems and to select others, particularly recombinant MSP-$3_{212-380}$ with AS02 adjuvant, based on antibody titres induced. One drawback of this choice is that the number of animals in each group, together with unavoidable variations in response from one animal to the other, does not allow us to reach statistically significant differences between groups. This was, however, compensated by performing a comparative analysis of antibody titres induced, particularly upon native parasite proteins, with the course of parasitaemia upon challenge. The FUP-SP strain of *P. falciparum* proved to be highly virulent for *S. sciureus sciureus*, as previously described [20], in all naïve animals except one that resisted longer to parasite growth but developed severe anaemia. In contrast, the immunized animals controlling parasite growth had higher antibody titres than those shown to be more susceptible, providing a strong indication that protection is associated with antibody levels above a given threshold and providing a clue as to why all animals within a group did not have the same outcome.

In the case of MSP-3, our protection data are in agreement with that recently reported by Hisaeda *et al.* [24]. Using Freund's complete adjuvant in *Aotus* monkeys, these authors demonstrated improved protection in animals immunized with MSP-3 as compared to the most effective to-date MSP-1 and observed a correlation with prechallenge anti-MSP-3, though not anti-MSP-1, antibody titres. Our work now brings forth the important indication that protection may also be induced using adjuvants that are acceptable for human use, particularly AS02, with which considerable experience in humans has accumulated. The combined information derived from the two studies brings hope that similar formulations can be effective in humans. This is important as it has been proposed that only Freund's complete adjuvant could induce protection against malaria challenge in *Saimiri* and *Aotus* monkeys [25]. This was supported mainly by experiments performed using MSP-1-derived formulations [26]. Our results suggest that this conclusion might be limited to MSP-1, though not to other antigens.

We have studied several distinct antigen-adjuvant formulations. Some of the polypeptides employed as immunogens were of very small size and therefore possibly suboptimally immunogenic. They were selected on the basis of satisfactory immunogenicity in rodents (unpublished data), which however, may behave differently from primates and covered several B- and T-cell epitopes identified in malaria-exposed populations. The majority of the formulations tested were shown to be immunogenic, with lasting responses in most cases. Even when serum antibodies could no longer be detected several weeks or months following the last administration of the immunogen, a typical secondary response could be elicited with a single further dose. In fact, as few as two or three immunizing doses were sufficient in most cases to induce the highest titres observed, but the subsequent booster immunizations were important to show the induction of lasting memory by the formulations evaluated. In addition, the recall effect provides indirect evidence of proper T-cell stimulation by the formulations (T-cell proliferation assays were not performed to avoid major bleedings of these small animals). These data are encouraging in view of the limitations of adjuvants available for human use. The most effective formulation used here had the AS02 adjuvant (formerly SBAS2), which has successfully passed several trials in humans [27], as it is also the case of Montanide ISA720 [28]. Although animals in one of the two AS02 groups had received IFA 18 months earlier, the protection in the group that did not receive it was similar, indicating adequate efficacy of AS02 alone. The apparent lower immunogenicity of MSP-3 in relation to GLURP is counterbalanced by the observations that lower amounts of anti-MSP-3 antibodies are necessary to have the same effect of anti-GLURP antibodies in ADCI assays [6] (unpublished material) as well as in *P. falciparum*-infected SCID mice [23]. This is also supported by the control of parasitaemia observed in the present study.

The antibody titres were quite variable from monkey to monkey within a given group, whatever the antigen or adjuvant used. These observations are crucial in view of the relationship between prechallenge antibody titres and outcome of infection. It would seem that antibodies must be present in sufficient quantity to keep parasitaemia at low levels until boosting by the infection leads to a powerful secondary response that eliminates the parasite. Results suggest that suboptimal titres may still partially contain the parasite growth, but as the presence of parasitaemia for long period leads to anaemia, this in turn precluded a long-enough follow-up. This observation also brings the concern that suboptimal titres may lead to controlled but persisting parasitaemia and induction of anaemia in post-vaccination primary infections; hence, optimal immunization strategies should be developed. The relevance of

Saimiri monkeys to humans in terms of the epitopes targeted by immune responses and immunogenicity is debatable [22, 25, 29], so that the differences and individual variations in immunogenicity of the most promising formulations studied here would have to be addressed in phase I clinical trials.

In the case of GLURP, the control of parasitaemia in only one of three immunized animals remains of debatable significance, as this may have occurred by chance rather than by the effective action of antibodies elicited by immunization. Again, two observations provide insight supporting the latter. Firstly, the protected animal presented antibody titres higher than the two nonprotected GLURP-immunized monkeys. Secondly, it presented a different pattern of fine epitope recognition. The peptides P3, P11 and S3 have been described as containing the major B-cell epitopes recognized by humans exposed to natural transmission [6], and affinity-purified human antibodies to P3 were shown to be the most effective in ADCI. In contrast, P5 and P11, two neighbouring epitopes in the $GLURP_{27-500}$ molecule, were found to be immunodominant for *Saimiri* monkeys. P3 was recognized only by the animal that controlled parasitaemia. This emphasizes the importance to study whether in phase I clinical trials $GLURP_{27-500}$ would induce antibodies with the same fine specificity as those induced under natural exposure conditions.

An important point is that the immunogens are well conserved among various *Plasmodium* isolates and that the challenge infection strongly boosted the specific response induced by immunization. Therefore, this can be also expected to occur in exposed vaccinated individuals.

In summary, the results reported here add a new piece to the data supporting MSP-3 and GLURP as malaria vaccine candidates and should stimulate further studies using larger groups of animals to confirm the results indicated by the present work.

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