

ANTIBODY RESPONSES TO *PLASMODIUM FALCIPARUM* SPOROZOITE-, LIVER- AND BLOOD-STAGE SYNTHETIC PEPTIDES IN MIGRANT AND AUTOCHTHONOUS POPULATIONS IN MALARIA ENDEMIC AREAS.

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Summary :

This study evaluates the differences in host immune responses to defined plasmodial antigens in four geographically different regions in which malaria is endemic. Sera from 527 individuals were tested for the presence of antibodies specific for three types of plasmodial antigen : liver-stage antigen (LSA-1), blood-stage antigen (SPF 70) and circumsporozoite (CS) antigen (NANP)₄. The individuals taking part in the study comprised : patients with transfusional malaria due to *Plasmodium falciparum* or *P. vivax*; non-immune migrants residing in an endemic area in Rondônia; Amazonian Indians from the states of Pará (Xingu PA) and Mato Grosso (Xingu MT); people living in a hyperendemic area in Africa (Burkina-Faso); and controls that had never been to a malaria endemic area. None of the transfusional sera displayed antibodies against sporozoite or to liver stage antigen, although 80% of the *P. falciparum* transfusional malaria sera contained IgG antibodies against the blood-stage peptide. A low percentage of Indians from Xingu PA and of non-immune migrants displayed antibodies against liver-stage (27% and 17%) and sporozoite (11% and 12%) peptides, although a greater frequency of antibodies against blood-stage peptide (50% and 49%) was observed in both cases. Indians from Xingu MT exhibited a greater frequency of antibodies against liver, sporozoite and blood-stage peptides (45%, 50% and 58%). Only hyperimmune African individuals exhibited higher percentages of antibodies against liver (64%) and blood-stage antigens (87%), contrasting with a low frequency of antibodies against the CS repeat (33%). Taken together, the present data confirm that Rondonian migrants and Indians from Xingu PA constitute populations with limited exposure and immunity to *P. falciparum* malaria infection and conversely, Xingu MT Indians and Africans have been more exposed to malaria infection. In conclusion this study indicates that the immune response to these malaria parasite peptides can be used to assess malaria transmission in epidemiological surveys.

Résumé : RÉPONSE ANTICORPS À DES PEPTIDES SYNTHÉTIQUES DES STADES SANGUINS HÉPATIQUE ET SPOROZOITE DE *P. FALCIPARUM* DANS DIFFÉRENTES RÉGIONS ENDÉMIQUES

Cette étude a évalué les différences de la réponse immune de sujets vivant dans quatre régions géographiques où le paludisme est endémique. Les sérums de 525 individus ont été testés vis-à-vis de trois peptides de *Plasmodium falciparum*, correspondant aux stades : hépatique (LSA-1), sanguin (SPF70) et sporozoite (NANP)₄. Les individus étudiés comprenaient des malades avec paludisme post-transfusionnel à *P. falciparum* ou à *P. vivax*, des migrants non immuns vivant à Rondonia (Brésil), des indiens brésiliens vivant dans les états du Pará (Xingu-PA) et du Mato Grosso (Xingu-MT), des africains résidant dans une région holoendémique de Garetanga (Burkina-Faso) et des sujets sains n'ayant jamais séjourné dans une zone endémique. Aucun des sérums de paludisme post-transfusionnel n'a réagi avec les peptides LSA-1 ou (NANP)₄ bien que 80% présentaient des anticorps pour le peptide SPF-70. Un faible pourcentage d'indiens du Xingu-PA et de migrants possédaient des anticorps contre les stades hépatique (27% et 17%) et sporozoite (11% et 12%) contrastant avec un pourcentage plus élevé d'anticorps contre le stade sanguin (50% et 49%). Les indiens du Xingu-MT portaient plus fréquemment des anticorps contre les stades hépatique, sporozoite et sanguin (45%, 50% et 58%). Les africains ont montré des taux élevés d'anticorps contre les stades hépatique (64%) et sanguin (87%) contrastant avec la faible fréquence d'anticorps contre le (NANP)₄ (33%). Ces résultats suggèrent que l'étude de la réponse immune contre ces peptides peut être utilisée comme indicateur du degré d'exposition des populations.

KEY WORDS : *Plasmodium falciparum*, peptide, vaccine, malaria.
MOTS CLES : *Plasmodium falciparum*, peptide, vaccin, paludisme.

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INTRODUCTION

The importance of the humoral immune response in the defense against *P. falciparum* infections was clearly demonstrated three decades ago by Cohen *et al.*, 1961. They showed that the transfer of immunoglobulins from immune adults to infected children induced a rapid reduction in parasitemia levels. These results were recently corroborated by an analogous study by Bouharoun-Tayoun *et al.*, 1990. One clear implication of these

studies is that the humoral immune status of a population should be characterized prior to vaccination field trials in order to facilitate both the choice of an appropriate target population and the subsequent analysis of vaccination results that are often difficult to interpret.

In Brazil, there has so far been little research on humoral immune responses to *P. falciparum* synthetic peptides, and existing studies have focused their attention on the prevalence of antiparasite antibodies (Tosta and Moura, 1986; Arruda *et al.*, 1989; Oliveira-Ferreira *et al.*, 1992). We therefore decided to study *P. falciparum*-specific humoral immune response in autochthonous and migrant populations from endemic areas in four geographically different regions in which malaria is endemic, as well as in individuals with transfusion induced malaria, in order to evaluate the applicability of using this approach to study the degree of exposure to malaria. Immune responses against whole parasite antigen (PA) were also evaluated for comparative purposes.

MATERIAL AND METHODS

POPULATION STUDIED

The subjects investigated corresponded to a representative sample of the populations studied. The enrolment criteria used were: to be a permanent resident of the tribe since birth (Indians and Africans) or to be a migrant from non endemic area and residing in the municipality of Ariquemes (Rondônia). All individuals gave informed consent to participate in the study.

1) The population of non-immune migrants lives in the state of Rondônia in the southwestern part of the Brazilian Amazon. According to the Brazilian Ministry of Health, malaria is endemic in the area, without any marked seasonal variation. Rondônia, which accounts for 50% of the nation's registered malaria cases, saw the implementation of federal government agricultural settlement programs and a surge in mining activity in the 1970s. As a result, immigrants were attracted from other states (in southern and northeastern Brazil) that were mostly malaria free. Many of these migrants have been living in the state for as little as 2-4 years (41%) and the great majority (80%) for less than 10 years.

2) The two Indian populations comprise individuals living a) in the state of Pará (near the Tucuruí dam) and b) in the state of Mato Grosso (in the Amazon Basin in northern Brazil). These two tribes are widely separated and offer the possibility of studying *P. falciparum* immune responses in isolated localities. The characteristics of each tribe have already been repor-

ted elsewhere (Arruda *et al.*, 1989). Blood sample collections were performed at the end of the rainy season, a period of high transmission.

3) The African population is chronically exposed to the risk of malaria infection. It resides in Garetanga-Burkina Faso, a village located near the village of Donse (Druilhe *et al.*, 1986, Druilhe and Khusmith, 1987).

4) The transfusional malaria cases corresponded to individuals that have never been in endemic areas. The *P. falciparum* cases were from individuals living in Rio de Janeiro and those of *P. vivax* from individuals living in Belo Horizonte (Minas Gerais state) – malaria free areas – and were therefore primeinfected individuals.

DETERMINATION OF *PLASMODIUM* SPECIES

Malaria diagnosis was performed by microscopic examination of 500 fields of Giemsa-stained thick and thin blood smears. Parasitemia was quantified by examination of at least 200 leukocytes in thick blood films.

SERUM COLLECTION

Serum samples were obtained from a total of 527 individuals, as follows: 187 from non-immune subjects; 236 from Indians living in Pará (117) or the Mato Grosso (119); 78 from hyperimmune African individuals; 11 from patients with post-transfusional malaria (6 infected with *P. vivax* and 5 with *P. falciparum*); and 15 from control individuals who had never been to an endemic area. All the serum samples were collected without any preference in terms of age, race or sex. For the analysis of the results according to age, the age distribution of each group was done in order to have a homogeneous number of individuals in each one (Table 1).

P. FALCIPARUM SYNTHETIC PEPTIDES

– (NANP)₄, this synthetic peptide corresponds to four repeats of the repetitive epitope of the CS protein of *P. falciparum* (Zavala *et al.*, 1986).

– LSA-1, which corresponds to a repetitive epitope, part of a 200kDa molecule that is specific to the liver stage of *P. falciparum* (Druilhe *et al.*, 1984; Guérin-Marchand *et al.*, 1987). Its sequence is: LAKEKLQG-QQSDLEQERLAKEKLQEQQSDLEQERLAKEKLQ and purity higher than 90%.

– SPF70, which corresponds to a schizont protein, is a 70kDa antigen degraded from a 120kDa precursor membrane protein in mature schizonts and increases in amount at the time of schizont rupture (merozoite release/reinvasion). Although this molecule derives

AGE GROUPS (years)	(NANP) ₄				LSA-1			
	RO	XPA	XMT	AF	RO	XPA	XMT	AF
	p/n	p/n	p/n	p/n	p/n	p/n	p/n	p/n
1-10	13/39	8/50	22/37	12/32	5/39	14/50	24/37	78/32
11-20	12/50	9/21	50/26	32/22	22/50	14/21	46/26	95/22
21-30	9/44	14/21	74/23	40/10	11/44	33/21	69/23	0/10
>30	13/54	16/25	67/33	78/14	26/54	60/25	51/33	29/14
Total	12/187	11/117	50/119	33/78	17/187	36/117	45/119	64/78

Table 1. – Frequency of anti-circumsporozoite (NANP)₄ and anti-liver-stage (LSA-1) antibodies according to age in different populations.

p : percent positives; n : total number of individuals; RO : Rondônia; XPA : Xingu Pará; XMT : Xingu Mato Grosso; AF : Africa.

from *P. falciparum*, it also cross-reacts with anti-*P. vivax* antibodies. The synthesis of SPf70 was done by copolymerization of equal amounts of four different sequences of the 70 kDa molecule termed : C2 – GQDEGEENEG; C3 – LVFLVQQPFLFVLWDQ NEKFPVFMGVYDP; C5 DQFFDANPNLFLLEPVEFDED and C10 – GRNGLGANTDQDDQLEDE (James *et al.*, 1993; Toebe *et al.*, 1993).

IMMUNORADIOMETRIC ASSAY (IRMA) FOR DETECTION OF ANTI-*P. FALCIPARUM* SPOROZOITE-STAGE ANTIBODIES

The IRMA was performed as already described (Oliveira-Ferreira *et al.*, 1992; Zavala *et al.*, 1986) using microplates (Dynatech Alexandria, Virginia, USA) coated with (NANP)₄ conjugate to bovine serum albumin (BSA; Sigma). Control wells were prepared by omitting the peptide from the coat procedure. The counts per minute (CPM) in control wells were subtracted from the CPM in corresponding peptides coated plates. A serum sample was considered positive when the cpm value was higher than the average CPM of the normal sera plus two standard deviations (314 CPM).

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF ANTI-*P. FALCIPARUM* LIVER-STAGE ANTIBODIES

The ELISA was performed in polystyrene microplates (NUNC MAXISORP) coated with 100µl/well of a 2µg/ml antigen solution in phosphate-buffered saline (PBS). The plates were incubated overnight at 4°C and the antigen solution removed. The plates were

washed with PBS-0.05% Tween20 (Sigma; PBS-T20) and saturated with PBS containing 2.5% skimmed milk overnight at room temperature (RT). After washing with PBS-0.02% Tween20, test sera diluted 1:100 in PBS solution containing 1.25% skimmed milk and 0.5% Tween20 were added to the wells in duplicate, in volumes of 100µl/well, and incubated at RT for 1 hour. The plates were then washed as described above and 100µl of peroxidase-conjugated anti-human IgG (Sigma), diluted 1:1000 in the same buffer as the one used for sera dilution, was added to each well. After incubation (1h - RT), the plates were washed again and 100µl/well of the substrate solution was added (3% of OPD - O-phenylenediamine – Sigma – in 0.05M citrate buffer, pH 5.1, containing 0.07% H₂O₂ – Merck). The reaction was stopped by addition of 50µl of 2N HCl to each well and the absorbance was measured at 492nm with a Titertek Multiskan ELISA reader. The average optical density (OD) of a one-hundred-fold dilution of normal sera plus 2 standard deviations was defined as the cut-off limit (0.184 OD).

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF ANTI-*P. FALCIPARUM* PEPTIDE BLOOD-STAGE ANTIBODIES

We used polystyrene microplates (CORNING) coated with 100µl/well antigen solution (5µg/ml) in tris-buffered saline pH 8.0 (TBS). The plates were incubated for 16h at 4°C and the antigen solution removed. The plates were rinsed three times (for 10 min each time, with vigorous agitation) and then once for 30 min at 37°C with 200µl/well TBS-0.5% Tween20 (T20) (Sigma), to block nonspecific bindings. After bloc-

king, 100µl of test sera (diluted 1:100 in TBS-0.05%-T20) were added to the wells in duplicate, in volumes of 100µl/well, for 2h at 37°C. The plates were then washed 3 times, for 10 min each time, in TBS-0.1%-T20. The remaining steps in this assay were similar to those used for the detection of liver-stage antigen. The cut-off value was 0.132 OD.

IMMUNOFLUORESCENT ANTIBODY TEST (IFAT) FOR DETECTION OF ANTI-*P. FALCIPARUM* WHOLE BLOOD-STAGE ANTIBODIES

We used *P. falciparum*-parasitized red blood cells cultured asynchronously *in vitro* (Trager and Jensen, 1976). The IFAT was performed according to the method described by Ferreira and Sanches, 1988 using blood smears containing 20 parasitized red blood cells per field and fluorescein isothiocyanate conjugated goat anti-human IgG (Sigma). A reciprocal titer of 20 or greater was considered positive.

EPIDEMIOLOGICAL STUDY

Data were analyzed according to sex and age in all the population studied. In Rondonian subjects, other parameters, such as the presence of circulating parasites at the time of serum collection, the number of previous malaria attacks, and the date of the last attack, were also considered. Statistical analysis was performed using the EPISTAT software for the Chi-Square test with Yates' correction with 95% confidence intervals.

RESULTS

MALARIA PREVALENCE

At the time of blood collection in the state of Rondônia the prevalence of positive thick blood smears among the 187 individuals studied was 11%. Eighty percent of these positives were due to *P. falciparum* and 20% to *P. vivax*. No individual carried both species.

The prevalence of positive thick blood smears in Indians from Xingu MT (119) was 4.2%, with three individuals carrying *P. falciparum* and two carrying *P. vivax*. The level of parasitemia was very low in positive individuals (usually below 0.02%). In Indians from Xingu PA (117), the prevalence of positive thick blood smear was also low (3.4%). *P. vivax* was present in all the positive individuals, and none of them was positive for *P. falciparum*. Among the African subjects, 65% carried blood-stage parasites, and the positivity was higher in children than in adults. *P. falciparum* was the most frequent species accounting for 95% of the positives.

ANTIBODIES AGAINST *P. FALCIPARUM* LIVER-STAGE PEPTIDE (LSA-1)

As was the case for antibodies against the sporozoite-stage peptide, none of the transfusional malaria sera presented IgG antibodies against the LSA-1 peptide. The frequency of antibody responses to this antigen in Indians from Xingu PA and non-immune migrants in Rondônia was low (27% and 17%, respectively). Indians from Xingu MT and hyperimmune African individuals displayed higher percentages (45% and 64%, respectively) (Fig. 1). No relationship was observed between anti-LSA-1 IgG antibodies and sex in any of the populations studied. Previous research, like the present study, has shown that the development of antibodies to (NANP)₄ is age-dependent (Oliveira-Ferreira *et al.*, 1992). To determine whether a similar correlation exists for the LSA-1 peptide, we stratified antibody-positive rates by age, since all our selected populations had a homogeneous distribution in all the age groups studied (1-10; 11-20; 21-30; and >30 years). The frequency of anti-LSA-1 antibodies was significantly higher among individuals above 20 years old in the group from Xingu PA ($p < 0.001$), above 10 years old in the group from Xingu MT ($p < 0.01$) and less than 20 years old in the African group ($p < 0.001$) (Table 1). Among Rondonian individuals, no correlation was observed between the frequency of anti-LSA-1 IgG antibodies on the one hand, and either the presence of circulating parasites or the date of the last malaria attack of malaria or age, on the other.

ANTIBODIES AGAINST *P. FALCIPARUM* SPOROZOITE STAGE-PEPTIDE (NANP)₄

None of transfusional sera displayed IgG antibodies against the circumsporozoite protein (CSP). The frequency of IgG antibodies against CSP in migrants from Rondônia and Indians from Xingu PA was low (12% and 11%, respectively). Indians from Xingu MT exhibited a higher frequency (50%), while African individuals showed an unexpectedly low frequency (33%) (Fig. 1). No significant correlation was observed between anti-CSP IgG antibody and sex in any of the populations studied. However, as before, these antibodies was significantly higher in individuals above 20 years of age from Xingu MT and Africa ($p < 0.001$) (Table 1). Detection of these antibodies was not related to the presence of parasites in the peripheral blood or to the level of parasitemia in Rondonian subjects.

ANTIBODIES AGAINST *P. FALCIPARUM* BLOOD-STAGE PEPTIDE (SPF70) AND AGAINST THE WHOLE PARASITE ANTIGEN (PA)

Four out of five individuals with *P. falciparum* transfusional malaria displayed IgG antibodies against the

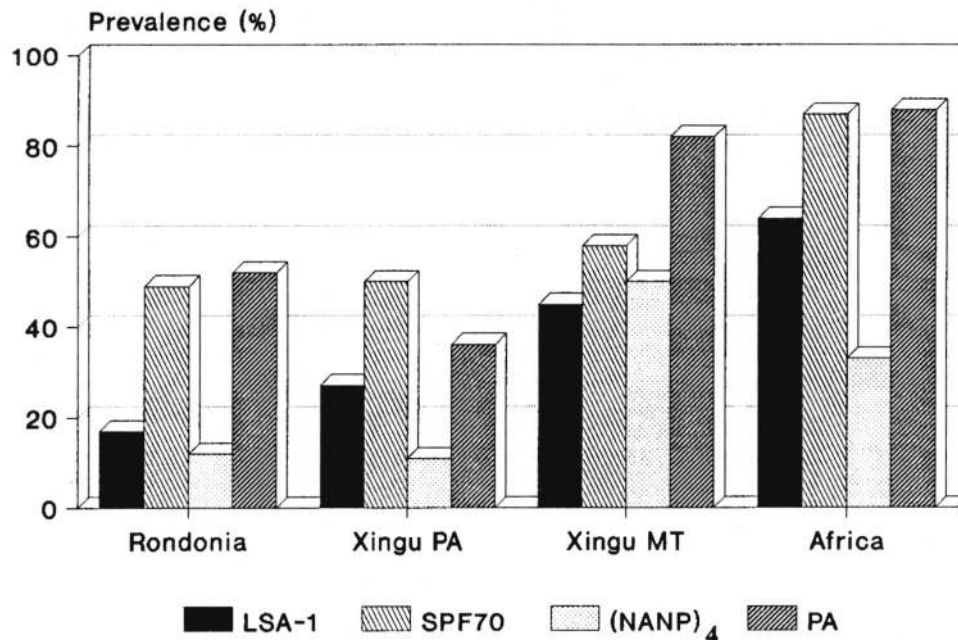


Fig. 1. – Prevalence of IgG antibodies against liver-stage (LSA-1), sporozoite (NANP)₄ and blood-stage (SPF70) and to blood-stage parasite antigen (PA) in different populations.

TM : transfusional malaria; RO : Rondônia; XPA : Xingu Pará; XMT : Xingu Mato Grosso; AF : Africa; PF : *P. falciparum*; Pv : *P. vivax*.

SPF70 peptide and against PA, as compared with only one (17%) *P. vivax* transfusional malaria case with antibodies against the SPF70 peptide. The frequencies of anti-SPF70 and anti-PA IgG antibodies were higher in individuals from Africa and Xingu MT (87%, 58% and 88%, 81%, respectively). Individuals from Rondônia and Xingu PA exhibited similar frequencies of anti-SPF70 IgG antibodies (50% and 49%, respectively). In Rondonians, the frequency of anti-PA IgG antibodies was also similar (52%) but in individuals from Xingu PA it was lower (36%) (Fig. 1). No relationship was observed between anti-blood stage antibodies and sex in any of the populations studied. The prevalence of IgG antibodies against PA was higher in the group aged over 10 years in Xingu PA. Among Indians from Xingu MT, the frequency of these antibodies increased with age, reaching 100% in individuals over 20 years old (Table 1). In the group from Rondônia, the frequency of anti-SPF70 IgG antibodies was significantly higher in individuals whose most recent malaria attack occurred within the last year (1 to 12 months) ($p < 0.001$). No significant correlation was observed between these antibodies and age in Rondonian individuals.

When antibody responses to the peptides and to PA were analyzed according to degree of malaria exposure, two main observations were made : a) the anti-LSA-1 antibody response is the only serological

marker that appears to indicate that the Xingu PA population has clearly been more exposed to *P. falciparum* than the Rondonian migrants; and b) conversely, the anti-(NANP)₄ response is the only antibody response which does not reflect the greater exposure of African individuals to *P. falciparum* malaria, as compared with other populations (Fig. 1).

DISCUSSION

In our study, none of the transfusional malaria (TM) patients had antibodies against (NANP)₄ or LSA-1, notwithstanding the high (80%) percentage of *P. falciparum* TM-infected individuals with antibodies against SPF70 and PA, and despite the data of Hope *et al.*, 1994, showing cross-reactivities between sporozoite and bloodstage antigens. This may be due to the relatively small number of TM cases in our study. Similarly, although antigen sharing between blood- and liver-stage schizonts has also been reported (Szarfman *et al.*, 1988a, 1988b; Suhrbier *et al.*, 1989), our data confirm the stage specificity of the liver-stage *P. falciparum* peptide used in the present study.

In Rondonian individuals, no correlation was found between the frequency of anti-(NANP)₄ antibodies and parasitemia. Similar observations were made by Oliveira-Ferreira *et al.*, (1992) in Brazil and Esposito

et al., (1988) in Africa, with both studies showing that the frequency of anti-sporozoite antibodies was not related to anti-blood-stage antibody titers or to reductions in parasitemia induced by chemoprophylaxis. Similarly, the absence of any relationship between the presence of anti-LSA-1 antibodies, the presence of circulating parasites and the time elapsed since the previous malaria attack suggest that these antibodies cannot be used as indicators of current or recent natural malaria infection.

In Indians from Xingu PA, the frequency of anti-CS protein antibodies was similar to that observed in the migrant population of Rondônia. Among these Indians, exposure to *P. vivax* is higher than to *P. falciparum*, in contrast to Indians from Xingu MT, who are more exposed to *P. falciparum*. This may explain why we found higher frequencies of antibodies against (NANP)₄, LSA-1 and PA in Indians from Xingu MT. Both the Xingu PA and Xingu MT Indian populations were visited at the end of the rainy season (December-April), a period of high transmission. Therefore, the difference in the frequencies of antibodies against (NANP)₄, a *P. falciparum*-specific antigen, in the two Indian populations could be explained by different degrees of exposure to *P. falciparum* and *P. vivax* rather than by seasonal variations as observed in other endemic areas (Esposito *et al.*, 1988; Druilhe *et al.*, 1986; Hoffman *et al.*, 1987; Deloron and Cot, 1990). Conversely, the similarity in the frequencies of anti-SPF70 antibodies observed in the two Indian populations probably reflects the fact that this peptide is recognized by antibodies from both *P. vivax*- and *P. falciparum* infected individuals (as demonstrated with the sera from transfusional malaria patients).

We observed a low frequency of anti-(NANP)₄ antibodies in the African population, in contrast to a high prevalence of anti-LSA-1 antibodies. This could indicate that in holoendemic areas, the immune response to the liver-stage peptide is a better indicator of malaria transmission than the response to the CS protein.

Among the populations in the present study, the African showed the highest frequency of anti-LSA-1 antibodies. In addition to the fact that this population was the most exposed to malaria, one other possible explanation for these data could be the existence of variant epitopes in the Brazilian strains of *P. falciparum*. This seems unlikely, however, since sequence data from two isolates have shown that the LSA-1 gene is well conserved (P. Druilhe, unpublished data). It seems that the low frequency of anti-LSA-1 antibodies among aged 21-30 or over Africans is inversely related to anti-sporozoite immunity, which increases progressively with age, being found in

around 80% of individuals over 30. This latter immunity may protect the population from the establishment of hepatic forms of the parasite. The African population also displayed the highest frequency of anti-SPF70 and anti-PA IgG antibodies. No significant correlation was observed between the frequency of antibodies against blood-stage antigens and age, in contrast to some previous studies on populations from endemic areas, (Deloron and Cot, 1990; Baird *et al.*, 1991), but in agreement with the study of Druilhe *et al.* (1986).

The present data confirm that Rondonian migrants and Indians from Xingu PA are characterized by limited exposure and immunity to *P. falciparum* antigens, and therefore should be considered a priority target for control measures in the future. Conversely, Xingu MT Indians seem to be more exposed to malaria infection and have probably reached a higher degree of immunity to *P. falciparum* antigens. In conclusion this study indicates that malaria parasite peptides can be used to assess malaria transmission in epidemiological surveys.

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