Abstract. A parasitophorous vacuole protein of Plasmodium falciparum, p126, is a potential candidate for a malaria vaccine. Its N-terminal region, composed of six repeats of eight amino acids, appears to be involved in the induction of protective immunity against P. falciparum in monkeys. This study evaluated the immune response to the N-terminal fragment of p126 (Nt47) in patients living in a malaria-endemic area of Brazil (Colina, Porto Velho, Rondonia). Cellular proliferative responses against Nt47 were low and infrequent. The study of the humoral immune response demonstrated that 95% of the patients had detectable anti-p126 antibodies and 77% had anti-Nt47 antibodies. Analysis of the antibody isotypes specific for Nt47 revealed that all four IgG subclasses were detectable in 77% of the samples. Patients who had a longer duration of malaria symptoms and a higher number of white blood cells had a lower percentage of anti-Nt47 antibodies. The results suggest that antibodies to the N-terminal region of the p126 protein may contribute to acquisition of immunity to P. falciparum malaria.

The Plasmodium falciparum p126 antigen is synthesized by the parasite during the asexual erythrocytic cycle. It is processed into fragments of 50 and 73 kD, the latter one composed of two peptides of 47 and 18 kD linked by disulfide bridges. This processing is associated with the release of merozoites from mature schizonts. The p126 protein, also known as serine-rich antigen or serine-rich protein, is based on the similarity of coding genes, ranks as a candidate antigen for inclusion in a subunit vaccine.

Patients. Forty-five patients diagnosed with P. falciparum malaria infection were followed. These subjects (33 males and 12 females with ages ranging from 11 to 62 years, mean = 26) were residents of Colina, a rural malaria-endemic village in Porto Velho municipality (Rondonia) in the southwestern part of the Brazilian Amazon. Since malaria transmission is low in this locality, blood samples were collected from individuals having malaria symptoms (active case detection) during the dry season (May–October), when most malaria infections occur. None of them were taking any prophylactic drugs. The majority (70%) of the subjects were immigrants from Brazilian nonmalaria-endemic areas and had been living in Colina for the last 10 years. Of the 45 patients, one was documented as having experienced a primary malaria episode. This migrant was from a nonendemic area, resided in the village for a short period of time, and had malaria symptoms for the first time. The other 44 had more than five episodes (60%). After obtaining written consent from all participants or from their parents and obtaining an oral history from the patient, 20 ml of blood were collected for both antibody analysis and an in vitro cell proliferation assay. Thin and thick blood smears were examined for identification of malaria parasites. The parasitemia was determined by counting parasites against a predetermined number of white blood cells in thick blood films. From this, the number of parasites per microliter of blood was calculated. Blood samples were collected on the day of diagnosis and at the beginning of treatment for seven days with quinine and tetracycline (day 0) and 16 days later (day 16). On day 0, the mean parasite density in patients was 11,400/μl of blood. At the time of the second bleeding (day 16), there were 15 subjects (33%) who had not yet cured the malarial
infection and presented low densities (mean = 90/μl) without clinical symptoms. Control samples were obtained from 25 individuals from the laboratory staff living in Rio de Janeiro with no history of malaria and who never visited areas with malaria transmission. Five patients with *P. vivax* infections were also included as controls. The study was reviewed and approved by the Ethical Committee of the Fundação Oswaldo Cruz.

**Parasite subcultures of isolates collected from infected patients.** The *P. falciparum* subcultures were performed using the method of Trager and Jensen. A 0.2-ml sample of heparinized intravenous blood collected from each infected patient was diluted 10-fold in culture medium consisting of RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 25 mM HEPES buffer (Sigma), 25 mM NaHCO₃ (Grupo Química, Rio de Janeiro, Baraí), 10 μg/ml of gentamicin (Boehringer, Mannheim, Germany), and 10% heat-inactivated AB nonimmune human serum. Aliquots of 0.5 ml of this preparation were distributed in 16-mm wells of flat-bottomed microtiter plates (Linbro, McLean, VA). The plates were placed in a candle jar and incubated at 37°C for 24–48 hr until schizonts were obtained. Cells were then washed five times in phosphate-buffered saline (PBS), pH 7.4, and resuspended at 30% hematocrit in PBS. Multispot slides were prepared with 5 μl of cell suspension per spot. Slides were dried and stored at -20°C.

**Monoclonal antibody.** The monoclonal antibody (MAb) 23D5 2H6 specific for the 50-kD proteolytic fragment of p126 used in the present study has been described previously. This MAb specifically reacts with the parasitophorous vacuole of *P. falciparum*-infected erythrocytes.

**Indirect immunofluorescence assay.** Indirect immunofluorescence assays (IFAs) were performed at 37°C in moist chamber after a 10-min fixation in cold acetone (-20°C). After incubation with MAb 23D5.2H6 (1:100 dilution), bound antibodies were detected by reaction with appropriate fluorescein isothiocyanate-conjugated antisera (Zymed, South San Francisco, CA).

**Immunoblot assay.** Immunoblot assays were performed using erythrocytes infected by *P. falciparum* schizonts and purified by centrifugation on metrizamide as the antigen. After washing in PBS, parasites were extracted in Laemmli sample buffer (62 mM Tris-HCl, pH 6.8, 2% [w/v] sodium dodecyl sulfate, 10% glycerol, 0.01% bromophenol blue) and electrophoresed in a 12% polyacrylamide gel containing sodium dodecyl sulfate. Antigens were transferred onto 0.45-mm pore sized nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Nitrocellulose bands were blocked in 15 mM Tris-HCl, pH 8.6, 140 mM NaCl, and 0.05% Tween 20 (TNT) buffer–5% nonfat milk, washed, and incubated individually with sera diluted 1:200 in TNT buffer. Bands were washed and incubated with appropriate peroxidase-conjugated antisera (Sigma) at a dilution of 1:1,000. After the bands were washed, the reaction was revealed with diaminobenzidine substrate (Sigma). The recognition of the p126 protein was carried out in comparison with controls tested with MAb 23D5.2H6 (1:100 dilution).

**Nt47 synthetic peptide.** A synthetic peptide corresponding to the repeat region of the amino terminus of p126 was prepared using a solid-phase method as previously described. This peptide was called Nt47 because of its localization at the amino terminus of the 47-kD subfragment of p126. The amino acid sequence of Nt47 is Thr Gly Glu Ser Gln Thr Gly Asn - Thr Gly Gly Gln Ala Gly Asn - Thr Gly Gly Gln Ala Gly Asn - Thr Val Gly Asn Gln Ala Gly Ser - Thr Gly Gly Ser Pro Gln Gly Ser - Thr Gly Ala Ser Pro Gly Gly Ser - Cys.

**Enzyme-linked immunosorbent assay.** The ELISA plates (high binding; Costar, Cambridge, MA) were coated overnight at 4°C with Nt47 peptide at a concentration of 2 μg/ml in 0.05 M NaHCO₃, pH 9.5. After blocking with 4% bovine serum albumin (BSA)/0.05% Tween 20 in PBS (1 hr at 37°C) and washing with deionized water-0.05% Tween 20, human sera diluted 1:100 in 1% BSA/0.05% Tween 20/PBS were added to the plates. After incubation (1 hr at 37°C), the plates were washed and appropriate peroxidase-conjugated anti-human IgG or IgM (Zymed) in 1% BSA/0.05% Tween 20/PBS was added and incubated for 1 hr at 37°C. To detect specific IgG subclasses, plates were incubated for 2 hr at 37°C with murine MAb labeled with peroxidase specific for human IgG subclasses (IgG1: PH6069; IgG2: HP6014; IgG3: HP6047; IgG4: HP6025; Zymed). Bound antibodies were detected with the substrate o-phenylenediamine (Merck, Darmstadt, Germany). The optical densities (ODs) were measured at 492 nm. Each sample was tested in duplicate. Sera from 25 nonexposed Brazilian donors were used to establish a normal range for the assay. The cut-off value was determined as the mean OD + 3 SD of Brazilian controls (Nt47 cut-off values: IgG = 0.220; IgM = 0.195; IgG1 = 0.195; IgG2 = 0.257; IgG3 = 0.427; IgG4 = 0.265). To standardize the OD data obtained in different experiments, for each immunoglobulin determination an OD index was calculated as the ratio of the OD observed/the cut-off value. Samples with an OD index greater than or equal to the following values were considered positive: IgG = 1.0; IgG1 = 1.6; IgG2 = 1.3; IgG3 = 4.1; IgG4 = 1.9; IgM = 1.5.

**Lymphocyte proliferation assay.** Peripheral blood mononuclear cells (PBMC) were purified by density gradient centrifugation (Ficoll-Hypaque). The PBMC cultures were performed in triplicate in 96-well, flat-bottomed microdilution plates at a concentration of 2 × 10⁶ cells per well in RPMI 1640 medium supplemented with 3.7 g/L of sodium bicarbonate, 2 mM glutamine, 10 mM HEPES buffer, 100 U/ml of penicillin and streptomycin, and 10% heat-inactivated human AB serum in the presence of Nt47 synthetic peptide (10 μg/ml), mitogens (phytohemagglutinin [PHA] or pokeweed mitogen [PWM]; 10 μg/ml), or purified protein derivative (PPD, 20 μg/ml). The PBMC were cultured at 37°C in a 5% CO₂ atmosphere for six days. One microcurie of ³H-thymidine was added to each well and incubated for at least 18 hr. Cells were harvested onto glass fiber filters and cellular incorporation of ³H-thymidine was measured by liquid scintillation counting. Stimulation indices (SIs) were calculated from geometric means of triplicate samples of stimulated wells divided by the geometric mean of unstimulated wells. A proliferative response was considered positive when the SI was greater than 2 and the difference between the geometric means of stimulated and unstimulated cultures was greater than 1,000 cpm. Blood samples of 12 individuals without a history of malaria who lived in down-
Town Porto Velho (an area considered free of transmission) were also processed.

Statistical analyses. The data were stored in the FoxPlus® (Borland International, Inc., Perryburg, OH) data bank software. Statistica (Microsoft, Inc., Redmond, WA) and Epi-Info 6 (Centers for Disease Control and Prevention, Atlanta, GA) statistical software programs were used for data analysis. The Student's t-test was used to analyze the differences in mean values, the chi-square test was used to analyze the difference in prevalence of positive responses, and the Spearman rank coefficient test was used to analyze the correlations between variables.

Results

Expression of p126 in isolates collected from P. falciparum-infected patients. It has been previously reported that the p126 protein is synthesized by P. falciparum between the 32nd and the 36th hr of the parasite erythrocytic cycle and is stored inside the parasitophorous vacuole. This means that p126 cannot be detected in the ring forms that are observed in the venous blood of P. falciparum-infected patients. To investigate whether the P. falciparum isolates collected directly from the 45 patients express p126, the isolates were subcultured until schizont forms appeared. Schizonts of all isolates (45 of 45) gave a parasitophorous vacuole specific fluorescence with the MAb 23D5.2H6. None of the P. vivax isolates (five) reacted with MAb 23D5.2H6 (Table 1).

Anti-p126 antibodies. Sera were tested for anti-p126 antibodies by Western blotting. At day 0, (day of diagnosis and at the beginning of malaria treatment) 36 (80%) of the 45 patients had antibodies that reacted with a 126-kD protein. Seven of nine patients who did not have detectable antibodies to p126 at day 0 became reactive at day 16 (time of the second bleeding), which increased the total number of responders to 43 (95%) of 45 (Table 1). Anti-p126 antibodies were not detected in sera from the 25 Brazilians not exposed to malaria (Table 1).

It must be emphasized that the two patients who did not have antibodies that reacted with the 126-kD protein were infected by P. falciparum isolates expressing the p126 protein (Figure 1). Moreover, it has been possible to detect antibodies that recognized a large number of other parasite antigens in their sera (Figure 2). No relationship was observed between anti-p126 antibodies and age, sex, reported number of previous malaria attacks, or time of residence in a malaria-endemic area.

Anti-Nt47 antibodies. The Nt47 synthetic peptide, which is based on the repeated sequence located in the N-terminal region of p126 (in the 47-kD subfragment), was used in an ELISA to detect specific IgG and IgM antibodies. There was an increase of the prevalence of IgG and IgM anti-Nt47 antibodies on day 16 compared with day 0, but this increase was not statistically significant (χ² = 1.3, P > 0.05, for both, Table 1). It appeared that eight of the 43 patients who developed antibodies against p126 did not have antibodies against Nt47. Ten of the 45 patients tested did not have (detectable) antibodies against Nt47 (the eight previously mentioned and the two who did not produce antibodies against the entire p126 antigen) (Table 1). However, all 10 patients had antibodies that recognized other parasite antigens (Figures 2 and 3). No differences were observed between the prevalence of anti-Nt47 IgG and IgM antibodies either at day 0 or day 16 (χ² = 0.05, P = 0.8, for both, Table 1). There was a positive correlation between the increase of IgG and IgM OD index from day 0 (r = 0.56, P = 0.0001) to day 16 (r = 0.36, P = 0.02). No relationship was observed between anti-Nt47 IgG or IgM antibodies and the parasite density either at day 0 (r = -0.10, P = 0.51; r = -0.24, P = 0.11, respectively) or at day 16 (r = 0.18, P = 0.28; r = -0.06, P = 0.70, respectively). No relationship was observed between IgG or IgM antibodies and age, sex, reported number of previous malaria attacks, or time of residence in a malaria-endemic area. Anti-Nt47 antibodies were not detected in the sera of 25 Brazilians not exposed to malaria (Table 1).

Anti-Nt47 IgG antibody subclasses. The subclasses of anti-Nt47 IgG antibodies were determined at days 0 and 16 for 28 of the 29 patients with detectable IgG-Nt47 antibody at day 0. The frequencies of Nt47 IgG1, IgG2, IgG3, and IgG4 antibodies on day 0 (68%, 54%, 50%, and 7%, respectively) were not statistically different from those observed on day 16 (71%, 65%, 52%, and 13%, respectively). The most prevalent anti-Nt47 antibodies were IgG1 followed by IgG2 and IgG3, but the difference between each subclass prevalence was not statistically significant (all, P > 0.05). In contrast, IgG4 antibodies against Nt47 were significantly less prevalent than IgG1 (χ² = 15.11, P < 0.0001), IgG2 (χ² = 11.76, P < 0.0006), and IgG3 (χ² = 6.70, P < 0.009).

### Table 1

<table>
<thead>
<tr>
<th>Individuals</th>
<th>Expression of p126</th>
<th>Antibodies anti-Nt47</th>
<th>Proliferative response to Nt47</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p126</td>
<td>Nt47 (IgG)</td>
<td>Nt47 (IgM)</td>
</tr>
<tr>
<td>Patients</td>
<td>Day 0</td>
<td>45/45 (100%)</td>
<td>29/45 (64%)</td>
</tr>
<tr>
<td></td>
<td>Day 16</td>
<td>Not done</td>
<td>35/45 (77%)</td>
</tr>
<tr>
<td>Controls</td>
<td>0/5 (0%)*</td>
<td>0/25 (0%)</td>
<td>0/25 (0%)</td>
</tr>
</tbody>
</table>

* P = 0.80 versus day 0 Nt47 (IgM).
† P = 0.05 versus day 0 p126.
‡ P = 0.17 versus day 0 Nt47 (IgG).
§ P = 0.80 versus day 16 Nt47 (IgM).
¶ P = 0.17 versus day 0 Nt47 (IgM).
# P = 0.25 versus day 0 proliferative response to Nt47.

** P. falciparum.

** P. vivax.
When we analyzed the OD index of cytophilic (IgG1 plus IgG3) to noncytophilic (IgG2 + IgG4) Nt47 IgG antibodies, we did not observe any significant variation between day 0 (7.4 ± 12.9 [mean ± SD]) and day 16 (6.8 ± 4.9). The relationship between the parasitemia and the OD index of cytophilic subclass was examined and the OD index could be classified into three categories: ≤ 4, > 4 and ≤ 8, and > 8. In these conditions, we observed that individuals with a high cytophilic subclass OD index (> 8) presented significant lower parasite densities at day 0 (P < 0.05) (Table 2). Such a correlation was not investigated at day 16 since patients were treated just after diagnosis (day 0). No relationship was observed between the OD index of Nt47 IgG subclasses or cytophilic subclass and age, sex, reported number of previous malaria attacks, or time of residence in a malaria-endemic area.

**Lymphocyte proliferation.** Lymphocyte proliferative responses were investigated in 30 of the 45 *P. falciparum*-infected patients. Lymphocytes from six (20%) of the 30 patients proliferated after stimulation with Nt47 peptide (Table 1). The prevalence of positive proliferative responses was higher on day 16 (6 of 30) than on day 0 (2 of 30) (Table 1). The mean ± SD delta cpm values for responders was 2,109 ± 868 cpm at day 0 and 3,290 ± 3,199 cpm at day 16. For all individuals, PBMC gave a high proliferative response to stimulation by the control antigen (PHA) (62,647 ± 20,965 cpm), and most of the patients also responded to PPD. No relationship was observed between proliferative responses and anti-Nt47 or anti-p126 antibody responses, sex, parasitemia, reported number of previous malaria attacks, or time of residence in a malaria-endemic area.

**DISCUSSION**

The aims of this study were to evaluate the natural occurring immune responses to p126 protein and to its N-terminal region in a group of *P. falciparum*-infected individuals from a malaria-endemic area in the Brazilian Amazon. We observed that 95% of patients had detectable anti-p126 antibodies independent of the reported number of previous malaria attacks or time of residence in a malaria-endemic area. We have already observed that immunologically naive persons experiencing their initial infections (French tourists visiting endemic areas) or chronically exposed individuals from different endemic areas (Senegal and Brazil) developed anti-p126 antibodies with a similar prevalence, which suggests that this protein is a good immunogen. However, anti-p126 antibodies were not detected in the sera of nine patients at day 0 and of 2 patients at day 16. The difference observed in these seven patients cannot be formally related to *de novo* production of anti-p126 antibodies following the infections, since these patients have already been infected (as shown by the presence of anti-*P. falciparum* antibodies in their sera at day 0; Figure 3). Therefore, it is possible that the lack of anti-p126 antibodies at day 0 is due to absorption of these antibodies by a relative excess
of the corresponding antigen. Alternatively, it is possible that p126-specific B cells were present, although circulating antibodies titers were undetectable. For this reason, only patients without detectable antibodies after treatment, i.e., at day 16, were considered as nonresponders.

The absence of anti-p126 antibodies in two patients is not due to a lack of p126 expression in the infecting *P. falciparum* since p126 was detected in both isolates by MAb. It is also unlikely that the lack of p126 antibody is related to the immunodepression observed in malaria-infected humans and experimental animals, since the p126 nonresponders had antibodies against other *P. falciparum* antigens. This absence of a response could be related to our previous observation that H-2b mice (but not H-2d H-2k mice) did not develop an immune response against the entire p126 antigen when immunized with *P. falciparum* schizont-infected erythrocytes.

In the present study, 23% of the patients did not have antibodies against the p126 N-terminal region (Nt47). Two possibilities could explain this lack of immune response in the patients. The first, based on the polymorphism of this domain of the molecule, results in a decrease in immune specificity. A deletion of one repeat of the N-terminal domain has already been described, which indicates that p126 can be expressed with five instead of six repeats of eight amino acids. Moreover, it has been observed that 88% of the *P. falciparum* isolates collected in the same area where we conducted our study contained six repeats and the remaining 12% contained five repeats (Zalis MZ, unpublished data). This first hypothesis does not explain the lack of Nt47 immune response since the possibility for one human (normally infected more than one time) to be exclusively infected by strains containing only five repeats instead of six is relatively low. In this regard, we have already observed by ELISA competition assay that human sera recognize peptides containing a minimum of four repeats (Ban-

<table>
<thead>
<tr>
<th>OD index</th>
<th>Mean ± SD parasites/μl (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤4</td>
<td>11,178 ± 11,850 (14)*</td>
</tr>
<tr>
<td>&gt;4 and ≤8</td>
<td>13,587 ± 10,209 (7)†</td>
</tr>
<tr>
<td>&gt;8</td>
<td>3,344 ± 2,896 (7)</td>
</tr>
</tbody>
</table>

* P < 0.05 versus >8.
† P < 0.05 versus >8.
Recent studies have demonstrated that antibodies to the N-terminal region of p126 are involved in protective immunity to \textit{P. falciparum} infection in monkeys.\textsuperscript{13, 14} However, it remains to be determined whether antibodies directed against the N-terminal domain of p126 contribute to anti-malarial immunity in humans. We observed that a high proportion of individuals had antibodies to the Nt47 peptide. The prevalences of IgG (77\%) and IgM (75\%) anti-Nt47 antibodies were similar. Although IgG anti-Nt47 antibody levels were higher in parasitemic than in nonparasitemic individuals, there was no relationship between the prevalence or levels of IgG or IgM anti-Nt47 antibodies and parasite levels, reported number of previous malaria attacks, time of residence in a malaria-endemic area, or date of the last malaria episode. It has already been reported that subjects protected against clinical malaria have high levels of cytophilic anti-plasmodial antibodies (IgG1 and IgG3).\textsuperscript{18} These antibodies could play a primary role in protection against malaria through antibody-dependent cellular inhibition (ADCI) of parasite growth and proliferation. However, this effector mechanism could be blocked by noncytophilic IgG2, IgG4, and IgM antibodies that have been found to be increased in the sera of unprotected subjects.\textsuperscript{19} In the present study, anti-Nt47 antibodies were found within all four IgG subclasses and individuals with a high OD index of anti-Nt47 cytophilic IgG antibodies (IgG1 + IgG3/IgG2 + IgG4) had low parasite levels, suggesting that these antibodies participate in the development of protection through ADCI. It has recently been suggested that the critical antigens that trigger ADCI are expressed on the merozoite membrane or at the apex,\textsuperscript{19} and the p126 has been described as an exported antigen processed into two fragments of 50 and 73 kD that are discharged as soluble proteins into the extracellular environment.\textsuperscript{1, 2} It is possible that these antigens, passively adsorbed onto merozoites, could have a potential role in triggering the ADCI mechanism as pointed out by Bouharoun-Tayon and others.\textsuperscript{19}

The finding of low and infrequent cellular proliferative responses against the Nt47 peptide in the group studied was rather surprising since high (SI = 6.7–36.7; mean ± SD = 14.7 ± 8.9) and frequent (54\%) proliferative responses were observed in the Dielmo (Senegal) group (Roussillon C, unpublished data). The difference in the pattern of proliferative responses between these two groups could be explained by the fact that the Dielmo group is composed of native subjects from an hyperendemic area, whereas the Brazilian group includes migrant subjects from an hypendemic area.\textsuperscript{26} Although the lack of proliferative responses in some subjects of both groups cannot be considered definitive evidence for a lack of T cell activation by Nt47 peptide, it could be related to 1) immunosuppression of peripheral T cells by parasite products, 2) reallocation of peripheral reactive T lymphocytes,\textsuperscript{37} 3) parasite antigenic polymorphism, and/or 4) polymorphism in the human major histocompatibility complex (MHC).

This field investigation on the immune response directed against p126 and its N-terminal region has shown that both are naturally immunogenic. Moreover, we observed that the high OD index of anti-Nt47 cytophilic IgG antibodies (IgG1 + IgG3/IgG2 + IgG4) were associated with low parasitemia, suggesting that cytophilic antibodies may participate in the development of protection against \textit{P. falciparum} infection. Studies in progress should be helpful in understanding the role of genetic polymorphism of the human MHC in the determination of the immune response to p126 and its amino terminal stretches.

Acknowledgments: We thank Marlene Mortaira for technical assistance and Genilson José Vieira for the photographs. We are grateful to all blood donors for participation in this study.

Financial support: This work received financial support from the cooperative program INSERM-FIOCRUZ and CNPq (Brazil). Dalma M. Banic, Joseli de Oliveira Ferreira, Lilian R. Pratt-Riccio, Debora Gonçalves, and Claudio T. Daniel-Ribeiro are recipients of a CNPq fellowship.


REFERENCES

expressing Plasmodium falciparum cDNA clones that are reactive with parasite inhibitory antibodies. Mol Biochem Parasitol 30: 9–11.


