HUMAN LEUKOCYTE ANTIGEN CLASS II CONTROL OF THE IMMUNE RESPONSE TO p126-DERIVED AMINO TERMINAL PEPTIDE FROM PLASMODIUM FALCIPARUM

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Abstract. We investigated the relationships between class II human leukocyte antigens (HLA) and the antibody response to Plasmodium falciparum p126 protein and to its amino-terminal portion (Nt47) in 2 malaria-endemic villages in Brazil, Colina and Ribeirinha. All people from the endemic areas had anti-p126 antibodies, and the frequencies of anti-Nt47 antibodies were similar in both communities (66% for Colina and 75% for Ribeirinha). Typing of HLA showed that Colina and Ribeirinha groups had no significant differences in HLA antigen frequencies. However, in both groups, significant associations between positive response to anti-Nt47 and presence of HLA-DR4, as well as between absence of response and presence of HLA-DR15, were observed. The predominance of positive responses to Nt47 among HLA-DR4 people was independent of the presence of any particular allele. There was no evidence for association between HLA-DQB1 alleles and antibody response to Nt47. Thus, naturally exposed people with different HLA class II antigens seem to respond differently to Nt47, indicating that the choice of relevant peptide sequences may have important consequences for subunit vaccine development.

INTRODUCTION

The p126 antigen is one of a number of erythrocytic-stage Plasmodium falciparum proteins being studied as potential malaria vaccine components. This protein has the capacity to induce protection against parasite challenge in various monkey species. Furthermore, it is expressed in all isolates and is highly conserved antigenically as well as structurally. The p126 protein, also known as serine-rich antigen 1 and serine-rich protein, is localized in the lumen of the parasitophorous vacuole and is also found adsorbed to the surface of free merozoites. Protein p126 fragments are released into the bloodstream at the end of the intraerythrocytic development phase of the parasite. The p126 protein contains protease domains, suggesting that it may have an essential function in merozoite release and reinvasion. The p126 amino-terminal portion, containing a 6-octamer repeat, has been shown to be involved in the induction of protection against P. falciparum challenge in monkeys. Furthermore, antibodies that react with this domain inhibit parasite growth in vitro. The p126 amino-terminal extremity was named Nt47 because of its localization at the amino-terminal end of the 47-kDa subfragment and is a potential target for vaccination protocols.

Previous studies demonstrated that Nt47 includes B- and T-cell epitopes recognized by infected humans. Analysis of the isotypic profile of the anti-Nt47 response has revealed that people with higher levels of specific cytophilic immunoglobulin (Ig) G antibodies had significantly lower parasitemia, suggesting that cellular effector mechanisms such as antibody-dependent cellular inhibition targeting the p126 antigen could play a primary role in the protection against malaria. We have also reported that 77% of malaria-infected people from the same endemic area presented detectable anti-Nt47 antibodies and that the lack of response in the remaining 23% could not be explained by the polymorphism present in some isolates that contained 5- instead of 6-octamer repeats. By use of congenic mice, we have shown that immune response to p126 protein is H-2 b and H-2 d restricted. This restriction can be overcome in cases where special constructs might also operate in humans. To test this hypothesis, an extended study on the Nt47 immunogenicity was conducted simultaneously with the characterization of the class II human leukocyte antigen (HLA) profile in 2 populations exposed to Plasmodium falciparum in endemic areas in the southwestern Amazon region in Brazil.

MATERIALS AND METHODS

Study subjects. The study was carried out in the farming area of Colina and the riverside fishing community of Ribeirinha, both near the state capital of Porto Velho (Rondonia) in the southwestern part of the Brazilian Amazon region. The population of Colina primarily consists of migrants from non-malaria-endemic areas in the south of the country and have been living in Colina for at least 10 years; the population of Ribeirinha consisted of natives from the Amazon Basin, with families that have lived in the community for at least 25 years. Plasmodium falciparum exposure and housing are similar in the 2 groups. Because malaria transmission is low in these localities, blood samples were collected during the dry season (May–October), when most of malaria infections occur. These samples were collected 4–5 times (5–7-month intervals) from each person in 1993 and 1994.

Samples were obtained from 50 inhabitants from Colina and 57 living in Ribeirinha. The Colina group consisted of 15
girls and women and 35 boys and men with ages ranging 8–59 years (mean, 31 years) at the beginning of the study. They stated that they had an average of 2 malaria episodes per year in the past 2 years. The Ribeirinha group consisted of 21 girls and women and 36 boys and men, ages ranging 10–74 years (mean, 31 years) at the beginning of the study. They stated that within the past 2 years, they did not have more than one malaria episode per year. Written informed consent was obtained from all donors or from their parents. The study was reviewed and approved by the Fundação Oswaldo Cruz Ethical Committee.

Blood was collected with ethylenediamine tetraacetic acid (EDTA) for both antibody analysis and HLA class II typing. Thin and thick blood smears were examined for identification of malaria parasites. Parasitemia was determined by counting parasites in a predetermined number of white blood cells in thick blood films, and the number of blood parasites per milliliter was calculated. Twenty-seven donors from Colina (54%) and 15 donors from Ribeirinha (26%) had detectable parasitemia at the time of blood sampling (P < 0.05). The mean parasite density—6,883 and 2,844/µL of blood in Colina and Ribeirinha donors, respectively—did not differ between the groups (P > 0.05). Patients were treated with quinine and tetracycline (Brazilian Ministry of Health’s conventional treatment for P. falciparum), and 20 days after treatment began, the people who had negative thick blood smears were included in the study. Blood samples from 78 donors living in urban areas of Porto Velho, where malaria transmission does not occur, were included in our study as normal malaria-endemic area controls. The Porto Velho group consisted of 44 women and 34 men, with ages ranging 20–63 years (mean, 38 years). Fifty-three percent of donors from Porto Velho were natives from the Amazon Basin, and 47% were from non-malaria-endemic areas in Brazil. They had all been living in Porto Velho for the last 22 years. All were negative for malaria parasites as assessed by thick blood films, and 70% denied prior malaria infection. However, 30% reported a mean parasite density—6,883 and 2,844/µL of blood in Colina and Ribeirinha donors, respectively—did not differ between the groups (P > 0.05). Patients were treated with quinine and tetracycline (Brazilian Ministry of Health’s conventional treatment for P. falciparum), and 20 days after treatment began, the people who had negative thick blood smears were included in the study. Blood samples from 78 donors living in urban areas of Porto Velho, where malaria transmission does not occur, were included in our study as normal malaria-endemic area controls. The Porto Velho group consisted of 44 women and 34 men, with ages ranging 20–63 years (mean, 38 years). Fifty-three percent of donors from Porto Velho were natives from the Amazon Basin, and 47% were from non-malaria-endemic areas in Brazil. They had all been living in Porto Velho for the last 22 years. All were negative for malaria parasites as assessed by thick blood films, and 70% denied prior malaria infection. However, 30% reported a mean parasite density of 1.4 ± 0.95 episodes of malaria occurring > 5 years before collection of samples. Further control samples were obtained from 25 people from the laboratory staff (Rio controls) who had both no history of malaria and who had never visited malaria transmission areas.

**Typing of DNA.** We extracted DNA from frozen peripheral blood nucleated cells by use of the phenol-chloroform extraction procedure. We performed HLA-DRB21 and DQB22 low-resolution typing by polymerase chain reaction with sequence-specific primers on all samples. In DR4-positive samples (55 alleles analyzed), polymerase chain reaction with DR4-specific primers for exon 2 amplification was followed by hybridization with sequence-specific oligonucleotide probes according to 12th International Histocompatibility Workshop protocols. Briefly, DR4-positive samples were amplified in duplicate by means of a forward primer and 2 different reverse primers for codon 86. Polymerase chain reaction products were checked in 1.2% agarose gel electrophoresis, and 3 µL of amplified denatured product was blotted onto replicate filters with a Hybridot manifold apparatus (Life Technologies, Gaithersburg, MD). Each oligonucleotide probe was labeled with 40 µCi [γ32P] adenosine triphosphate with T4 polynucleotide kinase. Sequences of the 13 probes for the most common DRB1*04 alleles were based on published sequences. Blots were hybridized overnight with probes at 54°C. Filters were washed twice in 2× standard saline citrate/0.1% sodium dodecyl sulfate (SDS) at room temperature, and once in TMAC solution (3 M TMAC, 50 mM Tris, 2 mM EDTA, 0.1% SDS, Denhardt 5×) for 45 min at 59°C. We reused each blot 2–3 times. To remove the probe blots were washed 3 times in hot (95°C) Tris-EDTA 10–10 (Tris pH 7.6 mM, EDTA 10 mM).

**Immunoblot assay for p126.** Immunoblot assays were performed by use of erythrocytes infected with P. falciparum schizosents purified by centrifugation on metrizamide23 as antigen. After washing in phosphate-buffered saline (PBS), parasites were extracted with a sample buffer (62 mM Tris-HCl, pH 6.8, 2% [w/v] SDS, 10% glycerol, 0.01% bromophenol blue) and electrophoresed on a 12% polyacrylamide gel containing SDS.25 Antigens were transferred onto 0.45-µm pore nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Membranes were blocked with TNT (Tris-HCl 15 mM pH 8.6, NaCl 140 mM, 0.05% Tween 20)-5% nonfat milk buffer, washed, and individually incubated with sera diluted 1/200 in TNT. After washing and incubation with the anti-human polyclonal immunoglobulin peroxidase-conjugates (Sigma, St. Louis, MO) at 1/1,000 dilution and further washing, the reaction was developed with diaminobenzidine substrate (Sigma). Identification of the p126 protein was carried out by comparison with reaction of 23D5.2H6 monoclonal antibody (1/100 dilution) specific for the 50-kDa proteolytic fragment of the p126 protein.

**Nt47 synthetic peptide.** A synthetic peptide corresponding to the repeat region of the amino-terminal end of the p126 protein was prepared with a solid-phase method as previously described. The amino acid sequence of this peptide is TGESQTGN-TGGQQAQN-TGGQQAQN-TGNOAGS-TGGSPQGS-TGASOPQSHC.

**Enzyme-linked immunosorbent assay for Nt47.** Enzyme-linked immunosorbent assay 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 Milli-Q water-0.05% Tween 20, human sera diluted 1% BSA/0.05% Tween 20/PBS (1:100) were added to the plates. After incubation for 1 hr at 37°C, the plates were washed, and the appropriate peroxidase-coupled anti-human IgG or IgM (Zymed, South San Francisco, CA) in 1% BSA/0.05% Tween 20/PBS was added and incubated for 1 hr at 37°C. Bound antibodies were detected with ortho-phenylenediamine (Merck, Darmstadt, Germany). Optical densities (OD) were measured at 492 nm. Each sample was tested in duplicate. Sera from 25 Rio controls were used to establish the normal range for the assay. The cutoff value was determined as the mean OD + 3 standard deviations of the Rio controls (Nt47 cutoff: IgG = 0.220; IgM = 0.195). To standardize the OD data obtained in different experiments, an OD index was calculated for each immunoglobulin determination as ratio of observed OD/cutoff values. Sample with OD index > 1.0 were considered positive.

**Statistical analyses.** Data were stored in the dBASE data bank software (Ashton Tate, Borland, CA). Statistica (Microsoft, Redmond, WA) and the EpInfo version 6 (Centers for Disease Control and Prevention, Atlanta, GA) statistical software programs were used for data analysis. Student’s t-test was used to analyze differences in mean values, and
Prevalence of antibody responses to pl26 and its amino terminal extremity (Nt47) in the samples from Colina, Ribeirinha, and Porto Velho groups under natural conditions of exposure. Five percent ing our earlier observation that this protein is a good immu-
on humoral responses to p126 and Nt47 are shown in Table 1. All people were tested for humoral responses against the p126 protein and Nt47 peptide. The longitudinal study of antibody response allowed us to analyze the correlation between variables. Antigen and gene frequencies were calculated respectively by the formula $af = n/N$, where $n$ is the number of samples positive for the antigen, $N$ is the total number of samples, and $gf = 1 - \sqrt{1 - af}$.27 The heterogeneity of HLA antigen frequencies among Colina, Ribeirinha, and Porto Velho groups and between responder and nonresponder groups was evaluated by chi-square test. The HLA antigen-specific associations with anti-Nt47 responders and nonresponders were analyzed by partition of total chi-square test.26 The HLA-unidentified specificities (blank) as well as HLA specificities with frequencies equal to zero in at least one of the studied groups were pooled into one group as other specificities.

RESULTS

Prevalence of antibodies reactive with p126 protein or Nt47 peptide. All people were tested for humoral responses against the p126 protein and Nt47 peptide. The longitudinal evaluation of p126 and Nt47 antibody responses provided a better identification of the responder group because the greater number of determinations (4–5 times) increased the possibility of detection in an area with low transmission rates. Data on humoral responses to p126 and Nt47 are shown in Table 1. Antibodies against p126 protein were found in all malaria-
exposed donors (100% for Colina and Ribeirinha), confirming our earlier observation that this protein is a good immu-

<table>
<thead>
<tr>
<th>Groups</th>
<th>Anti-p126</th>
<th>Anti-Nt47 (IgG and/or IgM)</th>
<th>Anti-Nt47 (IgG)</th>
<th>Anti-Nt47 (IgM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colina</td>
<td>50/50 (100)*</td>
<td>33/50 (66)†</td>
<td>23/33 (70)</td>
<td>29/33 (88)</td>
</tr>
<tr>
<td>Ribeirinha</td>
<td>57/57 (100)‡</td>
<td>43/57 (75)§</td>
<td>38/43 (88)</td>
<td>40/43 (93)</td>
</tr>
<tr>
<td>Porto Velho</td>
<td>5/78 (5)</td>
<td>4/78 (5)</td>
<td>1/4 (25)†</td>
<td>3/4 (75)</td>
</tr>
<tr>
<td>Rio de Janeiro</td>
<td>0/25 (0)</td>
<td>0/25 (0)</td>
<td>0/25 (0)</td>
<td>0/25 (0)</td>
</tr>
</tbody>
</table>

* $P < 0.0001$ versus Porto Velho group. † $P < 0.001$ versus Porto Velho group. ‡ $P < 0.001$ versus Porto Velho group. § $P < 0.01$ versus Porto Velho group. ¶ $P = 0.01$ versus Ribeirinha group.

The prevalence of anti-Nt47 antibodies was significantly higher in the Colina (66%) and Ribeirinha (75%) groups than in the Porto Velho group (Fisher’s exact test, $P < 0.001$ for both). No difference was observed in the anti-Nt47 antibody prevalence between the Colina and Ribeirinha groups (Fisher’s exact test, $P > 0.05$). The frequencies of anti-Nt47 IgG and IgM in Colina (70% and 88%, respectively) were not statistically different than those observed in the Ribeirinha group (88% and 93%, respectively). In contrast, in the Porto Velho group, the frequency of anti-Nt47 IgG antibodies was lower (25%) when compared with Ribeirinha (Fisher’s exact test, $P = 0.01$). Associations in both Colina and Ribeirinha of anti-Nt47 response with either age ($P = 0.04$ for both groups) or time of residence in a malaria-endemic area ($P = 0.04$ for both groups) were observed (Table 2). No relationship between responders and sex ($P > 0.05$ for Colina and Ribeirinha), reported number of previous malaria attacks ($P > 0.05$ for Colina and Ribeirinha), or parasitemia ($P > 0.05$ for Colina and Ribeirinha) was observed (Table 2). No anti-Nt47 antibodies were detected in serum samples from people (Rio controls) living in an area not endemic for malaria (Table 1).

The longitudinal study of antibody response allowed us to discriminate more precisely between responders and nonres-

### Table 1

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Responders</th>
<th>Nonresponders</th>
<th>Responders</th>
<th>Nonresponders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)*</td>
<td>34 ± 2.9</td>
<td>25 ± 3.2†</td>
<td>33 ± 2.6</td>
<td>25 ± 3.0‡</td>
</tr>
<tr>
<td>Male (%)</td>
<td>76</td>
<td>47</td>
<td>63</td>
<td>64</td>
</tr>
<tr>
<td>Female (%)</td>
<td>24</td>
<td>53</td>
<td>37</td>
<td>36</td>
</tr>
<tr>
<td>Time of residence (years)*</td>
<td>19 ± 2.4</td>
<td>11 ± 1.9‡</td>
<td>33 ± 2.6</td>
<td>25 ± 3.0§</td>
</tr>
<tr>
<td>Number of previous malaria attacks*</td>
<td>22 ± 3.4</td>
<td>15 ± 3.9</td>
<td>15 ± 2.5</td>
<td>12 ± 4.4</td>
</tr>
<tr>
<td>Parasitemia</td>
<td>6,193.3 ± 2,650.8</td>
<td>8,523.8 ± 4,792.3</td>
<td>676.4 ± 328.4</td>
<td>7,179.2 ± 6,955.3</td>
</tr>
</tbody>
</table>

* Mean ± standard error of the mean.
† $P = 0.04$ versus responders from Colina.
‡ $P = 0.04$ versus responders from Ribeirinha.
§ $P = 0.04$ versus responders from Ribeirinha.
On the basis of this observation, we regrouped anti-Nt47 data responder groups (chi-square frequencies were observed (chi-square shown in Table 3. No significant differences in HLA antigen responses to the Nt47 peptide.

Frequencies of HLA-DR antigens in Colina, Ribeirinha, and Porto Velho populations at Colina and Ribeirinha, respectively, seroconverted (Fisher’s exact test, P > 0.05 for both). We conclude that the prevalence of responders did not increase significantly from the first to the last (fourth or fifth) blood collection during the 2-year period. Three (6%) and 2 (3%) people at Colina and Ribeirinha, respectively, seroconverted (Fisher’s exact test, P > 0.05 for both). We conclude that nonresponders (an average of 30% of both groups) were those who, despite P. falciparum malaria reinfection and positive testing with the p126 antigen, did not have humoral responses to the Nt47 peptide.

**Class II typing**. Antigen frequencies in the 3 groups are shown in Table 3. No significant differences in HLA antigen frequencies were observed (chi-square = 34.054, df = 24; P = 0.0837). In Colina and Ribeirinha populations, the HLA-DR antigen distribution was found to be similar in both responder (chi-square = 16.385, df = 12; P = 0.174) and nonresponder groups (chi-square = 4.257, df = 9; P = 0.893). On the basis of this observation, we regrouped anti-Nt47 data in responder and nonresponder groups. The antigen frequencies for HLA-DR antigens in these 2 groups are shown in Table 4. Analysis of HLA-DR antigen frequencies in these groups revealed significant associations between anti-Nt47 response and the presence of HLA-DR4, and of HLA-DR15 with the absence of a response (chi-square = 5.218, relative risk [RR] = 3.871; P = 0.01 and chi-square = 8.581, RR = 0.158; P = 0.001, respectively, by partition of total chi-square test). Because the prevalence of anti-Nt47 seems to be related to age and exposure to malaria infections, we examined the possibility of DR15 people being younger or having inhabited the endemic area for a shorter period of time. However, no difference was observed in age (P > 0.05) or time of residence (P > 0.05) when we compared DR15 and DR4 people. Table 5 shows that HLA-DR4 allele subtyping was uninformative (chi-square = 7.766, df = 8; P = 0.456), and Table 6 shows that DQB1 allele typing accompanied the overall uninformative profiles in the groups analyzed (chi-square = 19.956, df = 18; P = 0.335), and no segregation with responding profile was evident.

**DISCUSSION**

Many studies conducted in the past years have searched for HLA-defined predisposition or influence in the pathogenesis of infectious diseases. Population-based studies have suggested associations of HLA antigens with susceptibility or resistance to leprosy, mucosal leishmaniasis, hepatosplenomegaly in schistosomiasis, and protection against ma-

### Table 3

Frequencies of HLA-DR in Colina, Ribeirinha, and Porto Velho groups

<table>
<thead>
<tr>
<th>HLA-DR</th>
<th>Colina (n = 50)</th>
<th>Ribeirinha (n = 57)</th>
<th>Porto Velho (n = 78)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.180</td>
<td>0.105</td>
<td>0.205</td>
</tr>
<tr>
<td>15</td>
<td>0.140</td>
<td>0.053</td>
<td>0.167</td>
</tr>
<tr>
<td>16</td>
<td>0.060</td>
<td>0.158</td>
<td>0.192</td>
</tr>
<tr>
<td>17</td>
<td>0.140</td>
<td>0.035</td>
<td>0.064</td>
</tr>
<tr>
<td>4</td>
<td>0.200</td>
<td>0.316</td>
<td>0.256</td>
</tr>
<tr>
<td>11</td>
<td>0.160</td>
<td>0.228</td>
<td>0.128</td>
</tr>
<tr>
<td>12</td>
<td>0.100</td>
<td>0.070</td>
<td>0.026</td>
</tr>
<tr>
<td>13</td>
<td>0.340</td>
<td>0.316</td>
<td>0.205</td>
</tr>
<tr>
<td>14</td>
<td>0.080</td>
<td>0.193</td>
<td>0.179</td>
</tr>
<tr>
<td>7</td>
<td>0.200</td>
<td>0.105</td>
<td>0.167</td>
</tr>
<tr>
<td>8</td>
<td>0.100</td>
<td>0.193</td>
<td>0.256</td>
</tr>
<tr>
<td>9</td>
<td>0.080</td>
<td>0.035</td>
<td>0.038</td>
</tr>
<tr>
<td>Other†</td>
<td>0.220</td>
<td>0.193</td>
<td>0.115</td>
</tr>
</tbody>
</table>

* Chi-square = 34.054, df = 24, P = 0.0837.
† HLA-DR18, HLA-DR10, and all unidentified specificities.

### Table 5

Frequencies of DR4 alleles in the studied groups

<table>
<thead>
<tr>
<th>DR4 alleles</th>
<th>Colina (n = 10)</th>
<th>Ribeirinha (n = 18)</th>
<th>Porto Velho (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>402</td>
<td>0.273</td>
<td>0.118</td>
<td>0.211</td>
</tr>
<tr>
<td>404</td>
<td>0.182</td>
<td>0.176</td>
<td>0.105</td>
</tr>
<tr>
<td>405</td>
<td>0.182</td>
<td>0.059</td>
<td>0.316</td>
</tr>
<tr>
<td>410</td>
<td>0.091</td>
<td>0.059</td>
<td>0.053</td>
</tr>
<tr>
<td>Other†</td>
<td>0.271</td>
<td>0.588</td>
<td>0.315</td>
</tr>
</tbody>
</table>

* Chi-square = 7.766, degree of freedom = 8, P = 0.456.
† Alleles 401, 403, 406, 407, 408, 409, 411, and 417.

### Table 4

Frequencies of HLA-DR antigens in anti-amino terminal extremity (Nt47) antibody responder and nonresponder groups

<table>
<thead>
<tr>
<th>HLA-DR</th>
<th>Anti-Nt47 responders (n = 70)</th>
<th>Anti-Nt47 nonresponders (n = 33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.145</td>
<td>0.129</td>
</tr>
<tr>
<td>15</td>
<td>0.039</td>
<td>0.226†</td>
</tr>
<tr>
<td>16</td>
<td>0.118</td>
<td>0.097</td>
</tr>
<tr>
<td>17</td>
<td>0.066</td>
<td>0.129</td>
</tr>
<tr>
<td>4</td>
<td>0.329†</td>
<td>0.097</td>
</tr>
<tr>
<td>11</td>
<td>0.158</td>
<td>0.290</td>
</tr>
<tr>
<td>12</td>
<td>0.066</td>
<td>0.129</td>
</tr>
<tr>
<td>13</td>
<td>0.316</td>
<td>0.555</td>
</tr>
<tr>
<td>14</td>
<td>0.145</td>
<td>0.034</td>
</tr>
<tr>
<td>7</td>
<td>0.184</td>
<td>0.065</td>
</tr>
<tr>
<td>8</td>
<td>0.184</td>
<td>0.065</td>
</tr>
<tr>
<td>9</td>
<td>0.053</td>
<td>0.065</td>
</tr>
<tr>
<td>Other†</td>
<td>0.197</td>
<td>0.161</td>
</tr>
</tbody>
</table>

* Chi-square = 8.581, P = 0.001.
† HLA-DR18, HLA-DR10, and all unidentified specificities.

### Table 6

HLA-DQB1 alleles frequencies in Colina, Ribeirinha, and Porto Velho groups

<table>
<thead>
<tr>
<th>HLA-DQB1 alleles</th>
<th>Colina (n = 50)</th>
<th>Ribeirinha (n = 57)</th>
<th>Porto Velho (n = 78)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.260</td>
<td>0.175</td>
<td>0.210</td>
</tr>
<tr>
<td>4</td>
<td>0.080</td>
<td>0.263</td>
<td>0.210</td>
</tr>
<tr>
<td>5</td>
<td>0.340</td>
<td>0.246</td>
<td>0.420</td>
</tr>
<tr>
<td>601-2</td>
<td>0.260</td>
<td>0.140</td>
<td>0.185</td>
</tr>
<tr>
<td>604-8</td>
<td>0.180</td>
<td>0.700</td>
<td>0.074</td>
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</tr>
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<tr>
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</tr>
<tr>
<td>9</td>
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<td>0.088</td>
<td>0.049</td>
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<tr>
<td>Blank†</td>
<td>0.140</td>
<td>0.088</td>
<td>0.160</td>
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* Chi-square = 19.956, degree of freedom = 18, P = 0.001.  
† Blank includes all unidentified specificities.
laria. Others have been unable to establish clear-cut correlations with different clinical forms in tuberculosis, malaria, schistosomiasis, leishmaniasis, and Chagas disease.

In malaria, restricted immune responses to parasite antigens have been shown in inbred mice. H-2^b mice respond to the repetitive domain (NANP) of P. falciparum circumsporozoite protein, and H-2^d mice respond to the glutamic acid-rich repeats of Pf155/RESA, and H-2^b and H-2^d mice are unable to respond to the p126 protein of P. falciparum. Although restrictions of the immune response to native proteins or to linear synthetic peptides can disappear when multiple antigen constructs are used, it is not yet clear whether the failure to respond is due to H-2 or to other non-major histocompatibility complex genes cosegregating in the different strains. On the other hand, responses in humans have been found to be promiscuous to some of these.

The association of HLA to immune responsiveness to malaria-defined antigens has been apparent in human vaccine trials. Low or undetectable responses to the synthetic peptide SP66 vaccine were found to be associated with HLA-DR4 or HLA-DR15/DQB1*0601 in different populations, and responsiveness to circumsporozoite-repeat vaccine (R32Tox-A) was found to be associated with HLA-DPB1*0501, a common antigen in the Thai population. These data highlight the importance of studying relationships between HLA and immune recognition of malaria vaccine candidate antigens before vaccine trials are conducted among people naturally exposed to the malaria parasite.

In previous studies, we found that in H-2^b and H-2^d mice unable to respond to the p126 protein, the restriction could be overcome by use of Nt47 synthetic peptide constructions. However, in preliminary studies of natural responses to the P. falciparum p126 protein, we observed that a percentage (23%) of the Colina population failed to develop antibody responses to its amino-terminal repetitive portion, the 49-amino-acid-long Nt47 peptide. Thus, to understand the basis of naturally acquired immune responses to Nt47, we have expanded our investigation to include another population of Amazon Basin natives (Ribeirinha) to verify whether the responses could be under the influence of specific HLA class II molecules.

All people from Colina and Ribeirinha had serological reactivity to the p126 protein, and a high proportion had antibodies against Nt47. In both groups, the prevalence of IgG and IgM anti-Nt47 was similar. Prevalence increased with age and time of residence in the endemic area, indicating that Nt47 antibody seropositivity increased with exposure, but there was no correlation of IgG or IgM anti-Nt47 antibody responses with the number of previous malaria attacks reported or with parasitemia levels. Nevertheless, in a previous study in which we examined the biologic activity of specific antibodies, we observed that anti-Nt47 cytophilic IgG of isotypes 1 and 3 could have an important role in the control of parasitemia loads in P. falciparum-infected humans.

The predominantly positive response to Nt47 observed in HLA-DR4 people was independent of the presence of any particular HLA allele. The small number of nonresponders carried the same alleles as their counterparts. The reason for the different level of response in these few people is not clear, but it might be explained by other intervening factors, such as other genes linked to the major histocompatibility complex or elsewhere.

To strengthen the notion that the different rate of response is applicable to any population, we analyzed samples from a third population with a different racial composition and from a different endemic area in the southwestern part of the Brazilian Amazon region (data not shown) and confirmed the responder bias in HLA-DR4-positive people.

The p126 protein and its amino-terminal portion elicit antibody responses in humans living in endemic areas and have been shown to contribute to parasite immunity. However, the data presented here reinforce the importance of ascertaining whether immunization will be restricted by HLA genes or would otherwise be able to induce protective immunity in all vaccinated people at risk, regardless of their HLA profile. Finally, these results lend further support to the need of developing multiepitope malaria vaccines to ensure efficacy and protection.

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