ANTIBODY RESPONSE PROFILES INDUCED BY *PLASMODIUM FALCIPARUM* GLUTAMATE-RICH PROTEIN IN NATURALLY EXPOSED INDIVIDUALS FROM A BRAZILIAN AREA ENDEMIC FOR MALARIA

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Abstract. The goal of this study was to evaluate the antibody response induced by *Plasmodium falciparum* glutamate-rich protein (GLURP) in naturally exposed individuals from the Brazilian Amazon region (Rondonia State). The results showed that most individuals had IgG against two well-defined regions within *P. falciparum* GLURP, the relatively conserved N-terminal nonrepeat region (R0) and the immunodominant repeat region (R2), 67% and 79%, respectively. The peptides S4 from R2 (53%) and P11 from R0 (49%) were identified as immunodominant B cell epitopes and induced higher levels of antibodies. The number of GLURP peptides recognized and the levels of IgG against S4 and P11 peptides showed a positive correlation with age and time of exposure in the malaria-endemic area studied. The antibody responses against GLURP epitopes appear to be modulated by HLA class II antigens. Interestingly, the GLURP immunodominant B cell epitopes in individuals from a Brazilian malaria-endemic area are distinguishable from those of the African malaria-endemic area. Considering the importance of GLURP as a malaria vaccine candidate and the increasing focus on the use of subunit vaccines in the control of infectious diseases, the concern of the influence of class II allele frequencies in ethnically diverse populations may be important before vaccine trials are conducted among people naturally exposed to malaria parasites.

INTRODUCTION

The *Plasmodium falciparum* glutamate-rich protein (GLURP) is an antigen considered to be one of the leading malaria vaccine candidates. GLURP is expressed in all stages of the parasite life cycle in humans, including on the surface of newly released merozoites. It is highly antigenic and the gene encoding GLURP shows little polymorphism in geographically different *P. falciparum* isolates. Moreover, previous immunoepidemiologic studies performed in high-transmission areas have shown a high prevalence of antibodies against GLURP in adults, as well as a significant association of high levels of GLURP-specific antibodies with low parasite densities and protection against clinical malaria. In addition, there has been evidence that cytophilic antibody responses to GLURP play a primary role in protection against *P. falciparum* malaria by effector mechanisms such as antibody-dependent cellular inhibition (ADCI), the cooperation of monocytes and antibodies impairing parasite multiplication. The relevance of the ADCI activity in naturally acquired immune protection against malaria has been previously described.

Earlier studies performed in areas highly endemic for malaria have demonstrated a high prevalence of antibodies against two well-defined regions within *P. falciparum* GLURP, the relatively conserved N-terminal nonrepeat region (R0) and the immunodominant repeat region (R2). Computer predictions of the most hydrophilic and antigenic domains of GLURP identified some potential B cell epitopes in regions R0 (P1, P3, P4, P5, P8, P9, P10, P11, and S3) and R2 (S4). The use of affinity-purified polyclonal antibodies from clinically immune adults living in areas highly endemic for malaria has identified six B cell epitopes in R0 (P1, P3, P4, P10, P11, and S3) and one in R2 (S4). Taking into account these regions, P3 and S3 of R0 appear to be the most important epitopes because human antibodies against them enabled the strongest ADCI effect in vitro.

Malaria in the Brazilian Amazon is hypoendemic to mesoendemic and is present throughout the year with seasonal fluctuations. Since *P. falciparum* and *P. vivax* exist simultaneously in this region, the exposed human population may have undergone several episodes of illness (by both species), symptomatic or asymptomatic, that varied in degrees of intensity with low reported mortality. Malaria parasites circulating in Brazil and South America may present immunologic characteristics different from those in Africa. In addition, Amazon inhabitants exposed to malaria, represented by native Amerindians, Caucasians, and mixed individuals, are ethnically different from Africans. Differences in host and parasite variability between Brazil and Africa are important factors in terms of vaccine strategies because the efficacy of a potential vaccine may vary in different epidemiologic scenarios. The goal of this study was to research in detail the antibody response against GLURP in malaria-exposed Brazilian Amazon individuals. The definition of the GLURP regions recognized by antibodies of such inhabitants may help to establish the best targets for a potential GLURP-based vaccine for future use by this population.

MATERIALS AND METHODS

Study site and subjects. This study was conducted in rural villages (Candeias do Jamari and São Miguel) near Porto Velho, the capital of Rondónia State, in the Brazilian Amazon malaria-endemic region. Transmission of malaria in Rondónia is hypoendemic to mesoendemic and is present throughout the year, with seasonal fluctuations with max-
mum transmission occurring during the dry season from May to October. The population in these two villages is composed of natives and Brazilian migrants inhabiting this area for variable times since the 1970s (mean ± SD time of residence in malaria-endemic area = 24.4 ± 16.3 years).

In the study period, the average annual parasitologic indices (annual positive blood slides per 1,000 population), an indicator of the probability of contracting malaria in the studied villages were 549, 677, 536, and 370, respectively, for 1993, 1994, 1995, and 1996. The Brazilian Ministry of Health considers high-risk areas those with an API ≥ 50. In Brazil, the confirmation of a malaria infection, and therefore its treatment, is strictly based on the parasitologic diagnosis (thick blood smear examination), not on febrile cases. *Plasmodium falciparum* and *P. vivax* occur simultaneously in these localities, but the predominant malaria parasite is *P. vivax*, representing 68%, 60%, 57%, and 66% of all malaria-registered cases, respectively, in 1993, 1994, 1995, and 1996. In the 1980s, the prevalence of *P. falciparum* exceeded that of *P. vivax* in these localities (Brazilian Ministry of Health, 2001, unpublished data). Serum samples were obtained from 187 malaria-exposed individuals (77 women and 110 men) with ages ranging from 8 to 74 years (mean ± SD age = 31.4 ± 15.8 years) at the beginning of the study (exposed group).

Serum samples from 109 individuals (61 women and 48 men with a mean ± SD age = 37.7 ± 10.2 years) living in downtown Porto Velho, where malaria transmission does not occur, were included in our study as non-infected individuals resident areas with no malaria in Porto Velho (Porto Velho group). This population was also composed of natives and Brazilian migrants who inhabited this area for various periods of time since the 1970s (mean ± SD time of residence in a malaria-endemic area = 26.5 ± 18.2 years). All individuals were negative for malaria parasites as assessed by thick blood films. Most (64%) denied prior malaria infection and 36% reported an mean ± SD of 1.8 ± 1.4 past episodes of malaria occurring more than five years before the collection of samples. Nonendemic control blood samples from 25 individuals of the laboratory staff (Rio de Janeiro, Brazil) who had no history of malaria or contact with areas of malaria transmission were included in the study as Rio de Janeiro controls. Signed consent was obtained from all individuals before admission to the study, which was reviewed and approved by the Ethical Committee of the Fundação Oswaldo Cruz.

Donors giving informed consent answered questions from an epidemiologic survey and blood samples were collected 3–5 times from each person during the dry season (between May and October), when transmission is higher. To evaluate the degree of exposure to malaria, the survey data used were age, time of residence in the malaria-endemic area, and the number of malaria episodes reported by each individual.

Blood was collected into tubes containing EDTA for both analysis of IgG and HLA class II typing. Thin and thick blood smears were examined for identification of malaria parasites. Parasitemia was determined by counting parasites in relation to a predetermined number of white blood cells in thick blood films, and the number of blood parasites per milliliter was calculated.

**Recombinant GLURP and synthetic peptides.** The production of recombinant proteins and synthetic peptides has been described in detail elsewhere. Recombinant proteins corresponding to the N-terminal non-repeat region GLURP_{24-489} (R0) and the C-terminal repeat region GLURP_{705-1178} (R2) of GLURP were used. In GLURP-R0-positive individuals, the samples were tested for humoral responses against peptides P3, P4, P5, P8, P9, P10, P11, and S3 covering known and predicted B cell epitopes within this region. In GLURP-R2-positive individuals, the samples were tested for humoral response against S4 peptide, corresponding to the R2 repeat unit.

**Enzyme-linked immunosorbent assay (ELISA) for recombinant proteins.** An ELISA with recombinant GLURP_{24-489} (R0) and GLURP_{705-1178} (R2) was performed as previously described. Microtiter 96-well plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4°C with 1 μL/mL of recombinant R0 or R2 (100 μL/well) in carbonate-bicarbonate buffer pH 9.6. Uncoated sites were blocked for two hours with 2.5% (w/v) powdered milk containing phosphate-buffered saline (PBS), and incubated for two hours at room temperature with sera diluted 1:100 in 1.25% (w/v) powdered milk containing 0.05% (v/v) PBS and Tween 20 (PBST). The plates were washed with PBST, mouse anti-human IgG (Sigma, St. Louis, MO) diluted 1:2,000 in 1.25% (w/v) powdered milk containing PBST was added, and the plates were incubated for one hour at room temperature. The plates were then washed with PBST, 100 μL of a solution of α-phenyldiamine and H_{2}O_{2} in citrate phosphate buffer, pH 5.0, was added to each well, the plates were incubated for 30 minutes at room temperature in the dark, and 50 μL of 2N H_{2}SO_{4} was then added to each well to stop the reaction. Plates were read at 492 nm in a spectrophotometer (Spectramax 250; Molecular Devices, Sunnyvale, CA). Sera from 25 Rio de Janeiro controls were used to establish the normal range for the assay. The cutoff value was determined as the mean optical density (OD) plus three standard deviations of the Rio de Janeiro controls (cutoff values: GLURP-R0 = 0.122, GLURP-R2 = 0.101). To standardize the OD data obtained in different experiments, an OD index was calculated for each immunoglobulin determination as the ratio of the observed OD to the cutoff values. A sample with an OD index > 1.0 was considered positive.

**Enzyme-linked immunosorbent assay for GLURP peptides.** An ELISA with synthetic GLURP peptides was performed as previously described. Plates were coated with 2.5 μg/mL of streptavidin (Sigma) in citrate-phosphate buffer, pH 5.0, overnight at 4°C. The plates were then washed with 0.5 M NaCl, PBS, 0.1% Tween 20, biotin-conjugated R0 (P3, P4, P5, P8, P9, P10, P11, and S3) and R2 (S4) peptides were added at a concentration of 1 μg/mL in 0.37 M NaCl, PBS, 0.1% Tween 20, and the plates were incubated for one hour at room temperature. The plates were washed with PBST, serum samples diluted 1:100 in 0.7 M NaCl, PBS, 0.1% Tween 20 were added, and the plates were incubated for one hour at room temperature. The plates were then washed with PBST, mouse anti-human IgG diluted 1:2,000 was added, and the plates were incubated for one hour at room temperature. The plates were washed with PBST and developed as in the ELISA for recombinant proteins. The cutoff value was determined as the mean OD plus three standard deviations of the Rio de Janeiro controls (cutoff values: P3 = 0.143; P4 = 0.148; P5 = 0.156; P8 = 0.143; P9 = 0.141; P10 = 0.151; P11 = 0.150; S3 = 0.149; S4 = 0.132). To standardize the OD data obtained in different experiments, an OD index was calculated for each
immunoglobulin determination as the ratio of the observed OD to the cutoff values. A sample with OD index > 1.0 was considered positive.

**HLA typing.** HLA typing was carried out in samples from 107 individuals naturally exposed to malaria and 77 individuals not exposed to malaria. Genomic DNA was isolated from frozen peripheral blood by a phenol-chloroform extraction procedure previously described.24 We conducted HLA-DRB3 and DQB2 low-resolution typing by a polymerase chain reaction with sequence-specific primers on all samples as previously described.25,26

**Statistical analysis.** Data were stored in dbASE databank software (Ashton Tate, Borland, CA). Statistica (Microsoft, Redmond, WA) and EpiInfo 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 chi-square analysis was used to compare the prevalence of positive responses. The Spearman rank coefficient test was used to analyze the correlation between variables. Antibigen frequencies were calculated by the formula af = n/N, where n is the number of samples positive for the antigen and N is the total number of samples, and gene frequencies were calculated by the formula gf = 1 - [(1 - af)^n].27 The heterogeneity of HLA antigen frequencies among rural and urban groups and between responder and non-responder groups was evaluated by the chi-square test. The HLA antigen-specific associations with anti-GLURP responders and non-responders were analyzed by the partition of total chi-square test.28 The unidentified HLA 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**

**Antibodies against R0 and R2 regions.** Individuals in the exposed group reported an average of 1.2 malaria episodes in the previous 12 months. All but 13 individuals reported at least one malaria episode, and 44 (23.5%) reported more than 10 episodes. Fortyeight individuals had detectable parasitemia (mean ± SD = 4,389 ± 9,610 parasites/μL) at the time of blood sampling, with 37 infected with *P. falciparum*, 9 with *P. vivax*, and 2 with mixed infections. The prevalence of IgG antibodies for GLURP regions R0 and/or R2 was 84% (157 of 187) in the exposed group. Antibodies to R2 were significantly more prevalent (79%) than antibodies to R0 (67%) ($\chi^2 = 5.94, P < 0.01$) (Table 1). Nine of the 13 patients reporting no history of malaria episodes had antibodies to GLURP. IgG antibody responses to both antigens were strongly associated with age (R0: $r = 0.39, P < 0.0001$ and time of residence in the malaria-endemic area (R0: $r = 0.01, P < 0.005$, R2: $r = 0.0005$, Table 1). The levels of IgG antibodies to R0 and R2 also increased with age (R0: $r = 0.28$, R2: $r = 0.30, P < 0.0001$ for both) and time of residence in the malaria-endemic area (R0: $r = 0.38$, R2: $r = 0.39, P < 0.0001$ for both). No relationship was observed between antibody responses to R0 and R2 and sex, presence or absence of the malaria parasites (whether *P. falciparum* or *P. vivax*) in blood, level of parasitemia, or reported number of previous malaria episodes (Table 1). The frequency of IgG antibodies to R0 and R2 in the Porto Velho group was low, 5% and 4%, respectively (OD index values: R0 = 1.767, R2 = 3.050). Some reactivity was expected because 36% of the individuals in the Porto Velho group reported previous malaria infection. However, none of the 25 Rio de Janeiro controls, who did have a history of malaria or contact with areas of malaria transmission, had detectable IgG antibodies against R0 or R2 regions (OD index values: R0 = 0.658, R2 = 0.702).

**Antibodies against GLURP-R0 and GLURP-R2 peptides.** We analyzed the IgG antibody responses against peptides P3, P4, P5, P8, P9, P10, P11, and S3 in the GLURP-R0 IgG antibody-positive individuals and against peptide S4 in the GLURP-R2 IgG antibody-positive individuals from the exposed group. The prevalence of IgG antibodies against R0 and/or R2 peptides was 65%. Twenty percent of these individuals recognized a single peptide, 34% recognized 2–5 peptides, and 11% recognized 6–9 peptides. The number of recognized peptides was higher in older individuals ($P = 0.0036$, $r = 0.2315$) and increase with time of residence in the malaria-endemic area ($P = 0.0001$, $r = 0.3614$), but was negatively associated with number of previous malaria episodes ($P = 0.0058$, $r = -0.2221$). As shown in Figure 1, individuals that recognized at least two peptides were significantly older ($P < 0.05$) and spent more time residing in the malaria-endemic area ($P < 0.05$).

The frequency of the IgG antibody response varied depending upon the peptide (Figure 2). The frequencies of response to P11 (49%) and to S4 (53%) were significantly higher compared with the other peptides ($P < 0.05$, for all). P11, S3, and S4 showed higher levels of IgG antibody re-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics of glutamate-rich protein (GLURP)-R0 and GLURP-R2 responders and nonresponders in the exposed group</th>
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<td></td>
<td><strong>GLURP-R0</strong></td>
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<tr>
<td></td>
<td>Responders</td>
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<td>Frequencies</td>
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<td>(67%)</td>
<td>(33%)</td>
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<td>Female (%)</td>
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<td>Age (years)†</td>
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<td>Time of residence in malaria-endemic area (years)‡</td>
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<td>Number of previous malaria attacks†</td>
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<tr>
<td>Parasitemia (parasites/μL)‡</td>
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* $P = 0.0145$, GLURP-R2 responders versus GLURP-R0 responders.
† Mean ± SD.
‡ $P < 0.05$, responders versus non-responders.
responses than did P3, P4, P5, P8 and P10 (P < 0.05 for all; Figure 2). Only the levels of IgG antibodies against P11 and S4 showed a positive correlation with age (P11: \( P = 0.001, r = 0.2816; S4: P < 0.0001, r = 0.3655 \)) and time of residence in the malaria-endemic area (P11: \( P = 0.0003, r = -0.2962; S4: P = 0.0002, r = -0.3062 \)). The high IgG antibody levels (>85th percentile of positive OD index from each peptide) observed in some individuals against different peptides (Figure 3) were not associated with any parameter analyzed in this study such as age, sex, time of residence in the malaria-endemic area, number of previous malaria episodes, or number of recent malaria attacks (\( P > 0.05, \) for all).

Two of the six Porto Velho group individuals that had IgG antibodies to GLURP-R0 had detectable antibodies against R0 peptides: one had antibodies against P3 (OD index value = 3.500), P4 (OD index value = 2.200), and S3 (OD index value = 1.500) and the other against P10 (OD index value = 1.300), P11 (OD index value = 1.500), and S3 (OD index value = 2.300). Three of the four Porto Velho group individuals that had IgG antibodies to GLURP-R2 had detectable antibodies against S4 (mean ± SD OD index value = 1.800 ± 0.608). However, none of the 25 Rio de Janeiro controls, who no history of malaria or contact with areas of malaria transmission, had detectable IgG antibodies to GLURP-R0 or GLURP-R2 peptides (OD index values: P3 = 0.458, P4 = 0.466, P5 = 0.524, P8 = 0.601, P9 = 0.425, P10 = 0.721, P11 = 0.689, S3 = 0.583, S4 = 0.633).

**HLA class II typing.** Most the individuals naturally exposed to malaria (107 of 187, 57%) and the Porto Velho unexposed individuals (77 of 109, 70%) were typed for HLA class II. There were no differences in HLA-DR and HLA-DQ antigen frequencies between these groups (\( \chi^2 = 23.376, \) degrees of freedom [df] = 14, \( P > 0.05 \) for HLA-DR; \( \chi^2 = 6.064, \) df = 7, \( P > 0.05 \) for HLA-DQ). To evaluate the effect of class II antigens on the immune responses to GLURP-R0 and GLURP-R2 peptides in the group exposed to malaria, we regrouped GLURP antibody data in the responder and non-responder groups. The individuals from the exposed group who were classified as non-responders were those who did not have detectable antibodies from the first to the last blood collection during the four-year period. Analysis of HLA-DR and HLA-DQ antigen frequencies in the responder and non-responder groups showed significant associations between these antigens and the immune response against GLURP-R0 and GLURP-R2 peptides. Table 2 shows significant associations between antibody responses to P3 and the presence of HLA-DR4 and HLA-DQ8; antibody responses to P4, P8, and P9 and the presence of HLA-DR13; and antibody responses to P10 and P11 and the presence of HLA-DR8; and antibody responses to S4 and the presence of HLA-DR7. Since the prevalence of antibodies to GLURP-R0 and GLURP-R2 peptides seems to be related to age, time of residence in the malaria-endemic area, and number of malaria episodes, the data for HLA-DR and HLA-DQ antigen associations with antibody profiles were analyzed taking into account the effects of these parameters. However, no effects were displayed (\( P > 0.05, \) for all).

**Figure 1.** Average age (years) and time of residence in a malaria-endemic area (years), and number of previous malaria attacks with respect to the number of recognized peptides in the exposed group. *\( P < 0.05 \) versus negative; **\( P < 0.05 \) versus one peptide.

**Figure 2.** Frequency of individuals from the exposed group with IgG antibodies to glutamate-rich protein (GLURP)-R0 and GLURP-R2 peptides. *\( S4 \) versus P3, P4, P5, P8, P9, P10, and S3, \( P < 0.005 \) for all comparisons; **P11 versus P3, P4, P5, P8, P9, P10, and S3, \( P < 0.005 \) for all comparisons.

**Figure 3.** Distribution of the IgG antibody response (optical density [OD] index values) against glutamate-rich protein (GLURP)-R0 and GLURP-R2 peptides in the exposed group. Short line = average; dotted line = 85th percentile; long line = OD index.
GLURP is currently considered to be an important vaccine candidate antigen for malaria vaccine trials. Several studies performed in areas of Africa and southeast Asia hyperendemic for malaria have demonstrated that IgG antibodies to GLURP are involved in protection against clinical \textit{P. falciparum} malaria.

Human IgG to GLURP may play a role in the development of clinical immunity because this antibody mediates a strong monocyte-dependent inhibition (ACDI) of parasite growth \textit{in vitro}. The primary goal of this study was to evaluate the IgG antibody response induced by \textit{P. falciparum} GLURP in individuals from a Brazilian Amazon malaria-endemic area showing a different degree of malarial endemicity from those mentioned previously. To identify the main B cell epitopes of GLURP in naturally exposed individuals living in this area, we selected synthetic peptides covering sequences of GLURP identified as potential B cell epitopes.

We found that most individuals from the exposed group had IgG antibodies against the relatively conserved N-terminal nonrepeat R0 (67%) as well as the immunodominant C-terminal repeat region R2 (79%). The prevalence and the levels of antibodies against R0 and R2 regions increased with age and time of exposure in the malaria-endemic area. These two parameters were strongly correlated ($P < 0.0001$, $r = 0.5234$). Different data have been reported for an African area (Mayrno population in Sudan) of low endemicity, where antibody responses to R2 were observed in only 16% of 28 donors. Surprisingly, similar statistics were reported in an African area of high endemicity. Antibodies to GLURP were found in 9 of 13 patients who did not report a malaria episode. This result could be explained by exposure to infective mosquito bites, followed by asymptomatic infection.

Curiously, no association was established between prevalence or levels of antibodies to R0 and R2 and the number of previous malaria episodes in our studied population. In the case of response to individual peptides, there was a negative association between these parameters, suggesting that the response decreased with exposure. This finding may reflect a limitation of donor-reported data, especially individuals born in the area who do not recall childhood infections. However, people living for a longer period of time in the region may have acquired some degree of immunity to clinical disease after experiencing a number of infections, therefore reporting less episodes of clinical malaria in the more recent years. Conversely, nonimmune individuals who were younger or living for a shorter time in the area may have experienced more clinical episodes recently, leading to this bias in the analysis. Recent studies in this area have reported a considerable number of oligosymptomatic or symptomless malaria infections among individuals with long-term exposure (median = 18 years) to regions of hypoendemic unstable malaria transmission. In this regard, our studied population has been living in the Amazon region for an average of 19 years. Most likely, giving the high prevalence of \textit{P. vivax} in relation to \textit{P. falciparum} in the area, the number of malaria infections does not necessarily mean stimulation of responses to \textit{P. falciparum} GLURP. Further studies in this area could help to clarify whether the presence of antibody responses against GLURP (R0 and R2) is associated with oligosymptomatic and asymptomatic malaria. This phenomenon is not the focus of the work presented here.

The proportion of R0 or R2 responders from the exposed group with detectable antibodies that recognized at least one of the nine peptides identified as potential B cell epitopes was 65%. Peptides S4 from R2 (53%) and P11 from R0 (49%) were identified as immunodominant B cell epitopes. In addition to being recognized at higher frequencies, they induced high levels of IgG antibodies. Interestingly, the immunodominance of the P11 peptide has also been demonstrated in \textit{Saimiri} and \textit{Aotus} (Alves FA and others, unpublished data) monkeys immunized with GLURP recombinant proteins. These results are distinguishable from a previous study conducted in clinically immune Liberian adults, in whom the most frequently recognized B cell epitopes of GLURP were P3 (55%) and P4 (48%). Moreover, in such a population, only 29% of the studied individuals had detectable antibodies against P11 (S4 was not assayed in this work). Two hypotheses may explain the differences in the immunodominance of GLURP peptides. The first involves genetic differences in the GLURP \textit{P. falciparum} between the isolates of the two malaria-endemic areas. As shown previously, a limited variation in the R0 region was observed when field isolates from different geographic locations (including Brazilian isolates) were compared, in contrast with the considerable polymorphism exhibited by the R2 repeat region. The second involves the interethnic differences between the two studied populations. Published studies have demonstrated the existence of marked differences in \textit{P. falciparum} rates, malaria morbidity, antibody responses to various \textit{P. falciparum} antigens, and genetic background, which probably involve genetic regulation of the immune responses.

### Table 2

<table>
<thead>
<tr>
<th>Antigens</th>
<th>HLA class II genotypes</th>
<th>Association with antibody response and HLA</th>
<th>Chi-square</th>
<th>Relative risk</th>
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*Pos. = positive association; neg = negative association.*

### DISCUSSION

GLURP is currently considered to be an important vaccine candidate antigen for malaria vaccine trials. Several studies performed in areas of Africa and southeast Asia hyperendemic for malaria have demonstrated that IgG antibodies to GLURP are involved in protection against clinical \textit{P. falciparum} malaria.

Human IgG to GLURP may play a role in the development of clinical immunity because this antibody mediates a strong monocyte-dependent inhibition (ADCI) of parasite growth \textit{in vitro}. The primary goal of this study was to evaluate the IgG antibody response induced by \textit{P. falciparum} GLURP in individuals from a Brazilian Amazon malaria-endemic area showing a different degree of malarial endemicity from those mentioned previously. To identify the main B cell epitopes of GLURP in naturally exposed individuals living in this area, we selected synthetic peptides covering sequences of GLURP identified as potential B cell epitopes.

We found that most individuals from the exposed group had IgG antibodies against the relatively conserved N-terminal nonrepeat R0 (67%) as well as the immunodominant C-terminal repeat region R2 (79%). The prevalence and the levels of antibodies against R0 and R2 regions increased with age and time of exposure in the malaria-endemic area. These two parameters were strongly correlated ($P < 0.0001$, $r = 0.5234$). Different data have been reported for an African area (Mayrno population in Sudan) of low endemicity, where antibody responses to R2 were observed in only 16% of 28 donors. Surprisingly, similar statistics were reported in an African area of high endemicity. Antibodies to GLURP were found in 9 of 13 patients who did not report a malaria episode. This result could be explained by exposure to infective mosquito bites, followed by asymptomatic infection.

Curiously, no association was established between prevalence or levels of antibodies to R0 and R2 and the number of previous malaria episodes in our studied population. In the case of response to individual peptides, there was a negative association between these parameters, suggesting that the response decreased with exposure. This finding may reflect a limitation of donor-reported data, especially individuals born in the area who do not recall childhood infections. However, people living for a longer period of time in the region may have acquired some degree of immunity to clinical disease after experiencing a number of infections, therefore reporting less episodes of clinical malaria in the more recent years. Conversely, nonimmune individuals who were younger or living for a shorter time in the area may have experienced more clinical episodes recently, leading to this bias in the analysis. Recent studies in this area have reported a considerable number of oligosymptomatic or symptomless malaria infections among individuals with long-term exposure (median = 18 years) to regions of hypoendemic unstable malaria transmission. In this regard, our studied population has been living in the Amazon region for an average of 19 years. Most likely, giving the high prevalence of \textit{P. vivax} in relation to \textit{P. falciparum} in the area, the number of malaria infections does not necessarily mean stimulation of responses to \textit{P. falciparum} GLURP. Further studies in this area could help to clarify whether the presence of antibody responses against GLURP (R0 and R2) is associated with oligosymptomatic and asymptomatic malaria. This phenomenon is not the focus of the work presented here.

The proportion of R0 or R2 responders from the exposed group with detectable antibodies that recognized at least one of the nine peptides identified as potential B cell epitopes was 65%. Peptides S4 from R2 (53%) and P11 from R0 (49%) were identified as immunodominant B cell epitopes. In addition to being recognized at higher frequencies, they induced high levels of IgG antibodies. Interestingly, the immunodominance of the P11 peptide has also been demonstrated in \textit{Saimiri} and \textit{Aotus} (Alves FA and others, unpublished data) monkeys immunized with GLURP recombinant proteins. These results are distinguishable from a previous study conducted in clinically immune Liberian adults, in whom the most frequently recognized B cell epitopes of GLURP were P3 (55%) and P4 (48%). Moreover, in such a population, only 29% of the studied individuals had detectable antibodies against P11 (S4 was not assayed in this work). Two hypotheses may explain the differences in the immunodominance of GLURP peptides. The first involves genetic differences in the GLURP \textit{P. falciparum} between the isolates of the two malaria-endemic areas. As shown previously, a limited variation in the R0 region was observed when field isolates from different geographic locations (including Brazilian isolates) were compared, in contrast with the considerable polymorphism exhibited by the R2 repeat region. The second involves the interethnic differences between the two studied populations. Published studies have demonstrated the existence of marked differences in \textit{P. falciparum} rates, malaria morbidity, antibody responses to various \textit{P. falciparum} antigens, and genetic background, which probably involve genetic regulation of the immune responses.
The HLA class II molecules play an essential role in stimulating the immune response by binding and presenting peptides to CD4+ T helper cells. Differences in HLA binding affinities may result in decreased binding of specific peptides and an inefficient peptide presentation to CD4+ cells. Since B cells need to interact with CD4+ T cells in germinal centers to be activated and rescued from apoptosis, the deficient peptide presentation may result in decreased cytokine production by CD4+ T helper cells and, consequently, decreased production of antibodies by B cells. Certain HLA class II alleles were previously found to be associated to immune responsiveness to malaria-defined antigens in both individuals naturally exposed to malaria infection, as well as those involved in human vaccine trials.

In view of these data, we have assessed a possible involvement of HLA class II molecules in the modulation of antibody specificity profiles induced by P. falciparum GLURP in the exposed group. Results reported herein indicate that HLA class II molecules have a degree of influence on production of antibodies against R0 and R2 peptides (Table 2). The IgG seropositivity to R0-derived peptides (P3, P4, P8, P9, P10, P11) were associated with HLA-DR4, HLA-DR8, HLA-DR13, HLA-DQ-4, and HLA-DQ8, whereas the lack of an IgG antibody response against the R2 peptide (S4) tended to be associated with HLA-DR7. These associations do not appear to be the result of distortion of HLA-DR and HLA-DQ antigen frequencies in our population because there was no difference in the overall phenotype distribution. However, antibody responses against R2 and R0 peptides varied in the individuals, even when they carry the same broad specificities. One must be aware that other genetic factors may also be involved in these antibody responses. A study carried out in Gambia reported that certain HLA class II antigens (DR4, DR7, DR8, DR9, DQ7, and DQ2) were associated with seropositivity to a GLURP fragment covering the R1 and R2 (but not the R0) regions. Given the increasing focus on the use of subunit vaccines in the control of infectious diseases, the concern of the influence of class II allele frequencies in ethnically diverse populations may be important before vaccine trials are conducted among people naturally exposed to malaria parasites.

The number of GLURP peptides recognized and the levels of IgG against S4 and P11 peptides demonstrated a positive correlation with age and time of exposure in our studied malaria-endemic area, as opposed to the number of previous malaria episodes. These results suggest that individuals with a greater repertoire of antibodies against GLURP peptides and high levels of IgG antibodies against the main B cell epitopes S4 and P11 have more protection against the clinical disease. In this context, it is important to highlight that after relatively few malaria infections, clinical disease immunity may develop at a level determined by intrinsic immune factors governed by the age of the host, since most of the individuals (70%) in our study were adults more than 19 years old.

In conclusion we have shown that S4 and P11 are the GLURP immunodominant B cell epitopes recognized by individuals from our Brazilian malaria-endemic area, and that IgG antibody responses to these peptides seem to be related to protective immune mechanisms in such a population. Because numerous reports have described the relevance of cytotoxic antibody responses (IgG1 and IgG3) to GLURP in naturally acquired immune response protection against P. fal-

Received October 5, 2004. Accepted for publication May 19, 2005.

Acknowledgments: We thank the villagers of Candéias do Jamari and São Miguel for their participation in the study, Dr. Pedro H. Cabello for help with the HLA statistical analysis, Rosilene Ramos Gonçalves for technical assistance, Dr. Mitchell R. Lishon for his careful review of the manuscript. We also thank the Fundação Nacional de Saúde/Ministério da Saúde, Brazil for providing facilities in the malaria-endemic area.

Financial support: This work was supported by Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (Brazil), and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil). The authors are recipient of research fellowships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Lilian R. Pratt-Riccio), and CNPq (Josué C. Lima-Junior, Joselí Oliveira-Ferreira, Cláudio T. Daniel-Ribeiro, and Dalma M. Banič).

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