ASSOCIATION OF THE IgG RESPONSE TO *PLASMODIUM FALCIPARUM*
MEROZOITE PROTEIN (C-TERMINAL 19 kD) WITH CLINICAL IMMUNITY TO
MALARIA IN THE BRAZILIAN AMAZON REGION

ÉRIKA MARTINS BRAGA, ROSA MARIA BARROS, TÂNIA AFONSO REIS, COR JESUS F. FONTES,
CRISTIANE GUIMARAES MORAIS, MARIA SONIA MARTINS, AND ANTONIANA URSINE KRETTLI
Departamento de Parasitologia, Universidade Federal de Minas Gerais; Belo Horizonte, Minas Gerais, Brazil; Universidad Federal
de Mato Grosso, Cuiabá, Mato Grosso, Brazil; Centro de Pesquisas René Rachou/FIOCRUZ, Belo Horizonte, Minas Gerais, Brazil

Abstract. The antibody response to the C-terminal 19-kD fragment of *Plasmodium falciparum* merozoite surface protein-1 (PIMSP1–19) was investigated in groups of subjects living in areas of Brazil with different levels of malaria transmission. The prevalence and the levels of IgG to PIMSP1–19 increased with the time of exposure and were positively correlated with the absence of clinical symptoms in parasitemic patients. The frequency of positive response and the mean level of IgG were higher in areas where malaria prevalence was more intense, especially among asymptomatic patients. The serum absorbance values of the IgG1 isotype were significantly higher among subjects with long-term exposure and in asymptomatic infections. These data suggest a protective role of IgG1 in naturally acquired immunity in spite of the unstable transmission levels in the Brazilian Amazon.

INTRODUCTION

Different studies have examined the efficacy of human antibodies in controlling the level of malaria parasitemia and several molecules have been characterized as target for such protection and as possible vaccines. One of the best-characterized antigens is merozoite surface protein-1 (MSP-1), a molecule with a molecular mass of approximately 200 kD that is expressed on the merozoite surface. During invasion of erythrocytes, this molecule undergoes a series of proteolytic cleavages resulting in four major fragments (MSP1–83, MSP1–28, MSP1–38, and MSP1–42). At the time of merozoite release, the MSP1–42 carboxy terminal fragment undergoes secondary processing to form a 33-kD product that is shed, whereas a 19-kD fragment (MSP1–19) remains on the merozoite surface during the invasion of erythrocytes. MSP1–19 is particularly interesting in vaccine development since naturally acquired antibodies to MSP1–19 from *Plasmodium falciparum* (PfMSP1–19) are associated with resistance to clinical malaria in hyperendemic areas. PfMSP1–19 has two domains, each with six cysteine residues that are characteristic of epidermal growth factor (EGF) motifs. The EGF domains in PfMSP1–19 are thought to be required in interactions with the erythrocytes. The use of allelic replacement to derive a transfected *P. falciparum* line that expresses a distinct MSP1–19 EGF domain from a rodent malaria parasite (*P. chabaudi*) showed that antibodies specific for this domain are a major component of the inhibitory response in immune humans.

It is well established that antibodies directed against the *P. falciparum* erythrocytic stage antigens, in addition to cell-mediated immunity, are important in immunity to malaria, and that passive transfer of sera has been shown to have a protective effect. However, the role of the IgG subclasses in the acquisition of antimalarial immunity is still unclear. *In vitro* and *in vivo* analyses of the effect of protective antibodies have shown that at least two mechanisms are involved: 1) opsonizing antibodies that promote phagocytosis of the infected erythrocytes and 2) cytophilic antibodies of human IgG1 and IgG3 isotypes that act in cooperation with monocytes to inhibit parasite growth. Epidemiologic studies in holoendemic areas in tropical Africa showed that cytophilic antibodies are associated with acquired protection, while non-cytophilic isotypes (IgG2, IgG4, and IgM) are predominant in non-protected groups. Thus, the isotypes or isotype balance rather than the levels of antibodies are important in antibody-mediated protection against malaria.

When one considers the crucial role of antibodies, especially the cytophilic antibodies, in controlling malaria parasitemia, it is relevant to characterize the pattern of antibody response to blood stage *P. falciparum* antigens and correlate the isotype composition of these antibodies in areas of different endemic levels.

Malaria in the Brazilian Amazon is hypoendemic to mesoendemic and transmission is unstable with seasonal fluctuations occurring throughout the year. The exposed population consists mainly of non-immune adults who are migrants from malaria-free regions. These individuals may experience several infections by *P. falciparum* or *P. vivax*, with clinical symptoms of variable degrees of intensity but low reported mortality (225 deaths in 2000). Since the prevalence of *P. falciparum* or *P. vivax* is rather variable within the endemic areas, we analyzed the natural antibody response to PIMSP1–19 using samples in which each species prevailed. The relationship between antibody responses to PIMSP1–19 and asymptomatic malaria among miners, with a long-term of malaria exposure, in one Brazilian endemic area was also examined.

MATERIALS AND METHODS

Study areas, subjects, and blood sample collection. We analyzed four groups of subjects who had been exposed to malaria transmission in the Brazilian Amazon endemic area and reported a variable number of previous episodes caused by *P. vivax* or *P. falciparum* with clinical symptoms (Table 1).

The first group lived in Belém, the capital of the state of Pará. It consisted of 18 individuals (median age = 31 years) who had acquired a single episode of *P. vivax* malaria after a brief exposure (a few days) on the island of Cotijuba, which is situated 25 km from the capital.

The second group lived in Cuiabá, the capital of the state of Mato Grosso, where no malaria transmission occurs. This group consisted of 49 adults (median age = 32 years) who had become infected through short visits to mining and/or...
Demographic and epidemiologic data of the subjects exposed to endemic malaria transmission in the Brazilian Amazon, and IgG responses to the C-terminal 19-kD fragment of Plasmodium falciparum merozoite surface protein-1 (PfMSP1–19)

<table>
<thead>
<tr>
<th>Characteristics (sample)</th>
<th>Groups (n)</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Belem (18)</td>
<td>Cuiaba (49)</td>
</tr>
<tr>
<td>Gender, male (%)</td>
<td>12 (67)</td>
<td>40 (82)</td>
</tr>
<tr>
<td>Median age (years)</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time of malaria exposure (mean ± SD)</td>
<td>&lt;1 month</td>
<td>&lt;1 year</td>
</tr>
<tr>
<td>Number of previous malaria episodes</td>
<td>1†</td>
<td>1–10</td>
</tr>
<tr>
<td>Exposure to malaria transmission/present malaria</td>
<td>Low/None</td>
<td>Sporadic/None</td>
</tr>
<tr>
<td>Number of positive sera for PfMSP1–19 IgG/total (%)</td>
<td>0/18 (0)</td>
<td>12/49 (25)</td>
</tr>
</tbody>
</table>

* Only cases of P. vivax were recorded.
† Only cases of P. falciparum were recorded.

Agricultural areas in the northern part of the state where malaria is endemic. They stayed less than one year in the endemic region.

The third group was composed of 97 subjects (median age = 30 years) who had resided for 10 or more years in Terra Nova do Norte, a small rural community within the endemic region. As a result, they were continuously exposed to the malaria transmission (median time = 10.5 years). This municipality had a total population of 20,000 inhabitants and the Ministry of Health reported 3,090 cases of malaria in the study year (1996). The total number of malaria cases reported in the municipality during the previous seven years showed that the highest malaria prevalence occurred in 1992, then gradually decreased.

These three groups consisted of aparasitemic individuals who were treated within two months prior to time of blood sample collection.

The fourth group consisted of migrant miners who had lived for approximately 20 years in several gold-mining areas in the Brazilian Amazon Basin. At the time of blood collection, these subjects were living in the municipality of Apiaxas, in the state of Mato Grosso, where approximately 1,200 cases/1,000 inhabitants were diagnosed in 1993; half of them were caused by P. falciparum.20 The prevalence of parasitemia decreased in subsequent years. In the study year (1996), the prevalence of malaria was 18% in the general population (531 miners evaluated), indicating that the pattern of transmission was mesoendemic.20 Among those miners characterized and followed-up in the area (by CJFF), we selected a parasitemic group composed of 99 subjects with P. falciparum or P. vivax infection. Two subgroups of these selected subjects were evaluated: 1) 46 subjects with symptomatic malaria, and 2) 53 individuals with no classical malaria symptoms for at least 72 hr after parasite detection. The symptomatic group reported mainly headache, anorexia, and fever. All miners were parasitemic at the time of first blood collection and remained parasitemic by Giemsa-stained thick-blood smears when re-examined 72 hr later in the case of the asymptomatic group.

All subjects were given a questionnaire that included information on past malaria and previous treatments. Consent for drawing blood was obtained from each individual according to the Fundação Oswaldo Cruz Ethics Committee (Ministry of Health, November 26, 1994) and the Universidade Federal de Minas Gerais Ethics Committee (April 15, 1998). Venous blood samples (20 ml/individual) were drawn in Vacutainer® heparinized tubes (Becton Dickinson, Oxnard, CA). Giemsa-stained thick blood smears were examined at this point.

Control subjects consisted of 40 healthy adult volunteers living in Belo Horizonte, in the state of Minas Gerais, who had never been exposed to the malaria transmission or visited the malaria-endemic region.

**Antigens.** The recombinant PfMSP1–19 protein, which represents the amino acid sequence of the EGF motifs of the Wellcome allele (MAD20 family) FVO strain, was expressed in Saccharomyces cerevisiae. The four main variable amino acids present in this recombinant protein were Q in the first motif and K-N-G in the second one.21 The protein was produced and kindly provided in 1997 by Dr. David Kaslow (National Institutes of Health, Bethesda, MD).

**Measurement of antibody levels.** An enzyme-linked immunosorbent assay (ELISA) for total IgG antibodies was performed as previously described.22 The concentration of PfMSP1–19 used was 1 µg/ml. All samples were diluted 1:160 and evaluated for total IgG using peroxidase-conjugated antihuman IgG antibodies (Sigma, St. Louis, MO). The optical densities (ODs) were measured at 490 nm and the threshold of positivity was an OD value of 0.235 based on the mean plus 3 SD of the reactivity of sera from 40 healthy controls who were not exposed to malaria transmission.

An ELISA to detect IgG subclasses was performed as previously described.23 Sera were diluted 1:50. The mouse monoclonal antibodies to human IgG or IgG subclasses used were clone HP-6012 for IgG1, clone HP-6014 for IgG2, clone HP-6010 for IgG3, and clone HP-6025 for IgG4 (Sigma). They were diluted according to the manufacturer’s specifications. Monoclonal antibody binding was detected with peroxidase-conjugated anti-mouse immunoglobulin (Sigma). The OD values were measured at 490 nm. The threshold of positivity was an OD value of 0.35 for IgG1, 0.52 for IgG2, 0.14 for IgG3, and 0.30 for IgG4. This was based on the mean plus 3 SD of the reactivity of serum from the 40 healthy individuals.

**Statistical analysis.** The Pearson correlation was used to assess the association between antibody levels and exposure to malaria. Differences in means were tested by the Kruskal-Wallis test. Differences in proportions were evaluated by the chi-square test using Epi-Info 6.03 (Centers for Disease Control and Prevention, Atlanta, GA). P values < 0.05 were considered significant.
RESULTS

Relationship of levels of antibodies against PfMSP1–19 to malaria exposure. The proportions of PfMSP1–19 IgG-positive subjects increased with exposure to malaria transmission (Table 1). It reached a peak of 88% in those subjects with long-term exposure and parasitemia at the time of blood collection (Apiacas group). The levels of IgG direct against PfMSP1–19 were also associated with exposure, since the greatest levels were observed in miners from the Apiacas group. Almost 40% of those sera showed OD values > 1.500 (Figure 1). No response was detected among the subjects who had experienced a single P. vivax malaria episode (Belem group) (Table 1).

The PfMSP1–19-specific IgG1 and IgG2 subclasses also showed differences related to exposure (Figure 2). Levels of the IgG1 isotype were significantly higher among the miners living for a long time in the endemic areas (Apiacas group) (Table 2). In contrast, the levels of IgG3 tend to be higher among subjects with short-term exposure to malaria transmission. Most sera tested had low levels of the IgG4 isotype (Figure 2 and Table 2).

Relationship between antibodies to PfMSP1–19 and asymptomatic infection. Serum samples from adult patients in the Apiacas group (Table 1) who had long-term exposure to transmission, 46 of which had clinical malaria and 53 with asymptomatic infections, were compared using an ELISA. A higher number of sera (98%) from subjects who experienced asymptomatic infections was positive compared with those with clinical manifestations (83%) (Table 3). Statistical analysis showed significant differences between the mean OD values for these two groups with regard to the presence of different isotypes against PfMSP1–19 (Table 3). A significant difference was found for IgG and IgG1-specific antibodies (P < 0.001). The subclass specificity of the PfMSP1–19 antibodies was predominantly IgG1 for both groups (Figure 3). IgG4 was detected only in six symptomatic patients (Table 3 and Figure 3).

DISCUSSION

The interaction between the host immune system and parasites varies according to the degree of endemicity. In areas of high endemicity, it has been assumed that acquisition of natural immunity to P. falciparum requires 10–15 years of uninterrupted exposure due to the antigenic polymorphism of the parasite. Protection from disease symptoms observed in older parasitemic children, known as clinical immunity, is usually never reached in regions of very low or seasonal exposure to malaria transmission. Among subjects lightly exposed in hypoendemic or mesoendemic areas, there is no association between age and severity of malaria. Such data support the hypothesis of a requirement for heavy and uninterrupted exposure to the sporozoite antigens and/or other parasite stages for protection from clinical malaria symptoms.

In different localities in the Brazilian endemic area with unstable transmission, we investigated the role of antibodies to the PfMSP1–19 antigen in naturally-acquired immunity. The individuals in the four areas studied were similar with respect to age and gender (mostly adult males). The prevalence of positive subjects and the levels of PfMSP1–19 IgG subclass antibodies to the C-terminal 19-kD fragment of Plasmodium falciparum merozoite surface protein-1 in three groups living in areas of Brazil with different levels of malaria transmission. For details on the four groups, see Materials and Methods.

![Figure 1](image1.png)

**Figure 1.** Percentages of individuals positive for IgG antibodies to the C-terminal 19-kD fragment of *Plasmodium falciparum* merozoite surface protein-1 in four groups living in areas of Brazil with different levels of malaria transmission. For details on the four groups, see Materials and Methods. OD = optical density.

![Figure 2](image2.png)

**Figure 2.** Percentages of individuals positive for various IgG subclass antibodies to the C-terminal 19-kD fragment of *Plasmodium falciparum* merozoite surface protein-1 in three groups living in areas of Brazil with different levels of malaria transmission. For details on the three groups, see Materials and Methods.

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Level of exposure to <em>P. falciparum</em></th>
<th>Absorbance at 490 nm (mean ± SD)<strong>†</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>Cuiabá (49)</td>
<td>Sporadic (&lt;1 year)</td>
<td>0.269 ± 0.429</td>
</tr>
<tr>
<td>Terra Nova do Norte (97)</td>
<td>Low/Constant (~10 years)</td>
<td>0.448 ± 0.675</td>
</tr>
<tr>
<td>Apiacas (99)</td>
<td>High/Constant (~20 years)</td>
<td>1.066 ± 0.832</td>
</tr>
<tr>
<td>Statistical analysis‡</td>
<td></td>
<td><strong>KW = 51.7</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

* For details of each group, see Table 1.
† Cut-off values are 0.34, 0.35, 0.32, 0.31, and 0.31 for IgG, IgG1, IgG2, IgG3, and IgG4, respectively.
‡ Kruskal-Wallis one-way analysis of variance.

TABLE 2

Effect of exposure to malaria transmission on levels of antibody to the C-terminal 19-kD fragment of *Plasmodium falciparum* merozoite surface protein-1 (PfMSP1–19)
were higher in the group exposed to long-term transmission in Brazilian endemic areas. Statistical analysis showed a positive association between time of exposure to an endemic region and high antibody levels to PiMSP1–19. Subjects who had had a single malaria episode caused by \textit{P. vivax} had no antibodies to the \textit{P. falciparum} MSP1–19 antigen, emphasizing the specificity of these antibodies. Analyses of IgG subclasses showed that the frequencies of PiMSP1–19 specific IgG1 and IgG2 were higher among subjects living in Apiacas, who had more malaria episodes. The level of specific PiMSP1–19 IgG1 was higher among those subjects with a long-term exposure to malaria when compared with subjects sporadically exposed. However, we reported a relative low IgG3 response in the former group concerning the intensity of the response (low mean OD values). The difference in IgG1 frequencies in aparasitemic individuals with one or many recent exposures confirms their ability to mount this response with limited malaria exposure. IgG3 has a serum half-life of only a few days. The IgG3 data among acutely infected miners from Apiacas group suggests that the significant lower level of antibodies to PiMSP1–19 in this group is an effect of intense exposure to malaria transmission.

In a study conducted in the Brazilian Amazon, the MSP1 IgG isotype distribution to recombinant proteins derived from polymorphic block 2, conserved block 3, and dimorphic blocks 6 to 8 was evaluated.\textsuperscript{26} The results showed that IgG3 to some peptides tended to increase among acutely infected patients with more frequent malaria exposure. The levels of antibodies to conserved and dimorphic blocks, but not to polymorphic block 2, decreased in such patients two months after the acute episode.

In the malaria-exposed populations, the low responsiveness of subjects from Cuia\ba and Terra Nova do Norte could be explained by the low levels of \textit{P. falciparum} transmission in these regions or by serologic restriction. Indeed, despite the conserved nature of PiMSP1–19, four amino acids changes in the EGF domains have been commonly found in natural isolates.\textsuperscript{27,28} In Brazilian (State of Rondonia) \textit{P. falciparum} populations, seven MSP1–19 variants were found and the QKNG haplotype was detected in approximately 40% of the 138 sequences obtained from 130 isolates collected between 1985 and 1998.\textsuperscript{29} Variant-specific responses cannot be excluded since it was not known to what extent the recombinant PiMSP1–19 used in this work represent the variants of the parasite population in the areas being studied.

An important question is whether antibodies to PiMSP1–19 are protective. The parasitemic subjects living in Apiacas offered an opportunity to correlate asymptomatic malaria and the IgG isotype distribution. Asymptomatic cases of malaria have been considered rare in Brazil.\textsuperscript{17,30} However, a study of miners in the state of Mato Grosso showed that 14% of the malaria cases were asymptomatic based on clinical examination and blood smear microscopy for diagnosis.\textsuperscript{31} We also observed that 11% of the malaria cases presently studied in 531 miners from Apiacas followed-up for two months and who had parasitemia caused by \textit{P. falciparum} or \textit{P. vivax} were asymptomatic. Asymptomatic \textit{P. vivax} malaria in a riverine Amazonian population has been demonstrated in the state of Rondonia in 14% of the subjects who were positive for \textit{P. vivax} by the polymerase reaction.\textsuperscript{32} These data demonstrate...
that acquired resistance to clinical malaria in Brazil occurs, as reported in Africa, despite the different epidemiologic profile of endemicity.

The high level of the PfMSP1–19-specific IgG1 subclass observed among the asymptomatic patients with clinical immunity suggests that IgG1 may mediate protective immune mechanisms. It is not clear whether anti-PfMSP1–19 IgG1 in those subjects exposed to malaria in Brazil is casually associated with the resistance to clinical disease. However, some studies conducted in hyperendemic areas showed that MSP1–19-specific IgG1, rather than IgG3, is associated with clinical protection from P. falciparum malaria.35–38 Younger individuals with high IgG1 responses tend to have lower parasite densities compared with individuals of the same age with negative or low responses.33 These observations support prior in vitro studies that indicate the existence of antibody isotype-dependent protection.15,16

In contrast to our results and those of other investigators, data from one recent study conducted in parasitemic infants and their mothers showed that the number of previous episodes of parasitemia since birth was negatively correlated with the IgG1 level to the 19-kDa antigen, suggesting that this response develops with age rather than with multiple exposures to blood stage parasitemia.36

The hypothesis of an age-dependent immunity to P. falciparum suggests that immunity may develop after relatively few infections to a level determined by intrinsic immune factors that change with age.37–39 The population evaluated in our study was composed of adults with various degree of exposure to unstable malaria transmission. The clinical findings associated with the IgG response to PfMSP1–19 in this population show that adults could acquire a semi-immune status after the exposure to unstable malaria in Brazil. In conclusion, we have shown that IgG1 and IgG2 isotype responses are predominant in subjects with long-term exposure to unstable malaria transmission and that high levels of IgG1 antibodies to PfMSP1–19 may be involved in asymptomatic infections. These data provide information on the characteristics of clinically acquired immunity in populations exposed to malaria transmission that could be beneficial in the development and testing of an MSP-1-based vaccine in different areas in Brazil where malaria is endemic.

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Authors’ addresses: Érika Martins Braga, Rosa Maria Barros, Tânia Afonso Reis, Cristiane Guimarães, and Maria Sonia Martins, Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, CP 486, 31270-901, Belo Horizonte, Minas Gerais, Brazil. Reprint requests: Érika M. Braga, Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, CP 486, 31270-901, Belo Horizonte, Minas Gerais, Brazil. Telephone: 55-31-34992876. Fax: 55-31-34992970, E-mail: embraga@icb.ufmg.br.

REFERENCES


