CELLULAR RESPONSES TO PLASMODIUM FALCIPARUM MAJOR SURFACE ANTIGENS AND THEIR RELATIONSHIP TO HUMAN ACTIVITIES ASSOCIATED WITH MALARIA TRANSMISSION

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Abstract. In Brazil, two types of activities have led to the worsening of malarial transmission in the Amazon region: prospecting/mining and agricultural settlements. In the present study, we analyze the cellular response of 52 of these individuals (14 gold-miners and 38 farmers) living within the same endemic area. Two Plasmodium falciparum major surface antigens (recombinant proteins) were used for cellular proliferative assays: circumsporozoite protein and merozoite surface protein-1. The frequency of these cellular responses were significantly higher among the miners (57–64%) than the farmers (10–20%) when either recombinant protein was used. Our data suggest that a higher exposure to malaria of the gold-miners contributed to their higher in vitro cellular response compared with the farmers. These findings point the way to further studies evaluating the influence of risk factors associated with the life styles of different social groups and the immune responses to these antigens.

Research directed towards developing an effective vaccine against Plasmodium falciparum has been conducted for decades.1 The surface antigens of sporozoites and merozoites are of particular interest because they represent two of the major candidates for inclusion in a subunit vaccine. The major constituents of the merozoite surface are polypeptides derived from a high molecular mass precursor protein known as merozoite surface protein-1 (MSP-1). Immunization with proteins derived from MSP-1 has been shown to protect animals from experimental infections, demonstrating its usefulness as a potential vaccine target.2-5

The best characterized of the anti-sporozoite vaccine candidates is the circumsporozoite (CS) protein.6 Initial clinical trials with the central repeat epitope of the P. falciparum CS protein, (NANP)6, resulted in a low level of protection.7,8 New clinical trials with the CS protein have been carried out in small number of subjects,3,10 but only one CS subunit vaccine was shown to be protective.11 Although these results represent a considerable advance in the development of malaria vaccines, it is still important to better understand the immune mechanisms involved in natural transmission, in which there are multiple parasite strains and the levels of transmission are variable.

In Latin America, malaria remains a serious public health problem affecting the lives and health of certain social groups, particularly those engaged in forest-related economic activities, gem mining, and work on road construction and hydroelectric dams. In Brazil, malaria is endemic in the Amazon area, which comprises 51% of the country’s territory and harbors only 9% of its inhabitants.12 Malaria has mainly affected the migrant populations coming from malaria-free areas of the country, attracted by opportunities for farming and mining.13,14 This was largely in response to the agricultural settlement projects of the National Institute of Colonization and Agrarian Reform, whose main emphasis was to start farms. Moreover, stimulated by the government migration policies and stricken by drastic impoverishment, large numbers of people moved in the 1980s to the Amazon Region looking for job opportunities in the gold-rich areas.15 Demographic analyses suggests that these new frontier areas are not retaining agricultural settlers, but attracting transient wage-laborers in ranching, mining, construction, and entrepreneurial activities such as gold mining or small businesses.16 In the Amazon, malaria prevalence is associated with occupational activities, indicating a predominance of outdoor transmission.17

We have undertaken a study of the natural immune response to CS protein in individuals living in different areas of the Brazilian Amazon. In the areas studied, the frequency of antibodies ranged from 11% to 43%, while the cellular response was detected in approximately 45% of the individuals.18 Moreover, in one area where the individuals were briefly exposed to a malaria outbreak, antibodies response against sporozoite and blood-stage antigens were detected up to seven years later.19,20 The present study focus on the cellular response of individuals living within the same malaria-endemic area but belonging to two different social groups, i.e., gold-miners and farmers. Recombinant P. falciparum proteins from the sporozoite (CS) and the blood-stage (MSP-1) were used for the in vitro lymphoproliferative assays; they represent two of the major candidates for inclusion in a subunit vaccine.7-11

MATERIALS AND METHODS

Area and individuals studied. The study was carried out in Pontes-Lacerda, Mato Grosso State, Brazil (Figure 1), which has one of the highest incidences of malaria in the Brazilian Amazon. In the year of the study, approximately 5,000 cases of the disease were reported in this region, where P. falciparum and P. vivax occur simultaneously.21

The 52 individuals studied (46 adults and six children 12–14 years old) were migrants from malaria-free areas of Brazil. They became infected while working in mining (n = 14) or agricultural (n = 38) activities. These two groups were treated separately in subsequent analyses. Epidemiologic and demographic data from the study subjects were collected by means of a questionnaire at the time the blood was taken. All individuals were questioned about their occupation, previous malaria experience, household structure, and time of
residence in the endemic area. The control group, which was never exposed to malaria, was composed of 16 healthy adult volunteers, 14 from the Research Center in Belo Horizonte (CPqRR/FIOCRUZ), Minas Gerais State, a nonendemic malaria area, and two from nearby cities in Minas Gerais where malaria never occurred.

The study was approved by the Ethics Committee of the Fundação Oswaldo Cruz (Brazilian Ministry of Health, Rio de Janeiro, Brazil). Informed written consent was obtained from each patient before participation in the study.

Antigens. The recombinant Plasmodium falciparum CS protein (rPfCS) was expressed in the yeast Saccharomyces cerevisiae transformed with plasmids containing DNA of the P. falciparum T4 isolate.22 This recombinant protein contains approximately 70% of the entire CS protein, including all of the repeats plus part of the flanking N- and C-terminal sequences of the protein (amino acids 43 to 391). The recombinant P. falciparum MSP-1 protein (rPfMSP-1), also expressed in S. cerevisiae, includes amino acids 120 to 435 of the N-terminal region of the protein (Uganda-Palo Alto strain).23 This recombinant protein is equivalent to the first 56% of the 83-kD N-terminal processing fragment.24 All recombinant antigens were produced by and obtained from the Chiron Corporation (Emeryville, CA). Control antigens included an extract of S. cerevisiae (Difco, Detroit, MI) and the mitogen phytohemagglutinin (PHA; Sigma, St. Louis, MO).

Blood sample collection. Venous blood samples were drawn into Vacutainer® (Becton Dickinson, Oxnard, CA) heparinized tubes after the individual’s permission was obtained according to the official FIOCRUZ Ethics Committee Rules (Brazilian Ministry of Health). Giemsa-stained thick blood smears from all individuals included in the study were negative at the time of sample collection. We selected only parasitologically negative individuals since the in vitro cellular response to malaria antigens is diminished during acute infections.25,26

In vitro proliferation assays. Peripheral blood mononuclear cells (PBMC) were isolated by Histopaque®-1077 (Sigma) density gradient centrifugation as described elsewhere.18 The PBMC were cultured in 96-well plates at a concentration of 1 × 10⁷ cells/ml in culture medium consisting of RPMI 1640 medium supplemented with 25 mM sodium bicarbonate, 25mM HEPES buffer, 3mM L-glutamine, 3% antibiotic-antimycotic mixture (300 U of penicillin, 300 µg of streptomycin, 0.75 µg of fungizone/ml), and 10% AB+ normal human serum. The recombinant proteins (rPfCS and rPfMSP-1) and yeast extract (control) were used at final concentrations of 10 µg/ml;18 the mitogen PHA was used at a concentration of 12.5 µg/ml. After incubation of the plates (37°C in 5% CO₂) for three days (PHA) or six days (antigens), the cells were pulsed for 12 hr with 1 µCi/well of methyl-³H-thymidine (specific activity = 6.7 Ci/mmol; New England Nuclear Products, DuPont, MA). Cells were then harvested and radioactivity incorporation was determined by liquid scintillation. For the recombinant proteins, the specific stimulation index, expressed as SI, was calculated as the mean counts per minute (cpm) of antigen-stimulated cultures minus mean cpm of the yeast extract control, divided by the mean cpm of unstimulated cultures. The baseline level of positivity was an SI ≥ 2.5.

Statistical analysis. All statistical analysis was performed using the Epi-Info 6 software (USD, Inc., Stone Mountain, GA). Differences in means were tested by the Student’s t-test. Proportions were tested by the Yates’ Chi-square (χ²) or Fisher’s exact tests. P values < 0.05 were considered significant.

RESULTS

We investigated the cellular response to CS and MSP-1 proteins by proliferative assays in 52 individuals working either in mining (n = 14) or agricultural (n = 38) areas of the Brazilian Amazon (Pontes-Lacerda, Mato Grosso State). These two groups were comparable by age, sex, time of residence in the area, and time elapsed since their last malaria episode (Table 1). Their previous malaria experience, as assessed by verbal histories, showed a variable number of infections. To avoid extreme values and the possibility of recall bias, individuals who reported more than 10 previous malaria episodes were considered as having 11 episodes. The only significant difference observed between farmers and miners was the mean ± SD number of previous malaria episodes (4.5 ± 3.3 and 8.0 ± 3.6, respectively) (Table 1).

The distribution of the SIs using PBMC from the miners, farmers, or controls in response to recombinant proteins and to control antigens (PHA and yeast extract) is shown in Figure 2. The mean SIs for both recombinant proteins were significantly higher among the miners than the farmers. The PBMC from the individuals living outside the endemic area (controls) did not proliferate when stimulated with either recombinant protein (SI ≤ 2.0). All groups responded equally well to the mitogen PHA.

The SIs for the extract of S. cerevisiae were usually low in all groups (Figure 2). Only five of 68 individuals studied (three farmers and two controls) had somewhat reactive
TABLE 1
Demographic and epidemiologic data of the individuals studied in the State of Mato Grosso, Pontes-Lacerda County, Brazil

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Miners</th>
<th>Farmers</th>
<th>χ² = 0, P &gt; 0.05</th>
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<tbody>
<tr>
<td>Sex, male/total (%)</td>
<td>9/14 (64)</td>
<td>26/38 (67)</td>
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</tr>
<tr>
<td>Average age ± SD (years)</td>
<td>29 ± 9</td>
<td>27 ± 14</td>
<td>t = 0.64, P &gt; 0.05</td>
</tr>
<tr>
<td>Time of residence in the endemic area, years (mean ± SD)</td>
<td>5.2 ± 5.4</td>
<td>4.0 ± 3.3</td>
<td>t = 1.06, P &gt; 0.05</td>
</tr>
<tr>
<td>Previous malaria episodes (mean ± SD)</td>
<td>8.0 ± 3.5</td>
<td>4.5 ± 3.3</td>
<td>t = 2.9, P &lt; 0.05</td>
</tr>
<tr>
<td>Time elapsed in months since last malaria episode (% individuals)</td>
<td>&lt;1 (64)</td>
<td>≥1 (42)</td>
<td>χ² = 0.01, P &gt; 0.05</td>
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</table>

PBMC upon stimulation with the yeast (SI = 2.0–3.0). Nevertheless, we calculated the specific SIs for each individual by deduced values obtained with the yeast-stimulated cultures (Table 2).

The overall frequency of individuals responding to the P. falciparum antigens rPfCS and rPfMSP-1 was 23% and 38%, respectively (Table 2). Among the 38 farmers, 10% had a proliferative response upon stimulation with rPfCS, whereas among the miners, 57% had responding cells. In addition, the magnitude (mean ± SD) of the response was higher among the miners (2.9 ± 1.7) than the farmers (1.0 ± 1.5). Similarly, the PBMC from the miners show a higher frequency (64%) of cell proliferation in response to rPfMSP-1 and intensity, as measured by the mean ± SD SI (3.9 ± 3.2), than those from farmers (20%, mean SI = 2.0 ± 2.7) (Table 2).

DISCUSSION

The pattern of malaria transmission in Brazil is different from that of the hyperendemic areas of Africa and Southeastern Asia. Malaria in the Brazilian Amazon is unstable; its transmission fluctuates seasonally, being sustained by a low vectorial capacity, compared with areas of stable malaria. Consequently, clinical immunity to the disease is rarely seen, even in adults. In addition, the distribution of malaria in the Amazon region is not homogeneous. It is concentrated in areas with uncontrolled establishment of new rural and mining settlements and is associated with precarious dwellings and favorable work transmission conditions. The present epidemiologic situation gave us the opportunity to investigate the cellular immune response in two social
groups, gold-miners and farmers, living within the same endemic area of Mato Grosso State.

In the present work, of the 52 individuals studied, 23% and 38% had PBMC responses to rPfCS and rPfMSP-1, respectively. These data are consistent with our earlier studies that demonstrated that 30% of the individuals studied in the Brazilian Amazon had positive in vitro cellular responses to rPfCS. The only other report concerning cellular responses to MSP-1 among Brazilians was to P. vivax. The investigators used polypeptides representing the N- and C-terminal regions of the protein and showed PBMC stimulation ranging from 17% to 47% in the malaria-exposed group.

In the present study, the cell reactivity to the antigens rPfCS and rPfMSP-1 was considered parasite specific since the cellular response against both recombinant proteins were detected only in malaria-exposed individuals. However, we did not observe a lymphoproliferative response to these proteins in a large proportion of the Brazilian individuals who had had malaria previously. This result was not unexpected since it has been frequently reported that peripheral blood T cells from malaria-sensitized individuals have no or low responses to parasite antigens. This unresponsiveness is most apparent during acute infection. However, since all individuals included in our study were parasite free at the time of sample collection, we can exclude that this immunosuppression was due to an acute infection.

In view of the polymorphic nature of the CS and MSP-1 proteins, it is possible that antigenic diversity within T cell epitopes may contributed to this widespread lack of cellular response. An alternative explanation would be that the target antigens of an efficient memory T cell response are poorly immunogenic. Genetic restriction could also contribute to a poor immune response. Recently, an association was demonstrated between malaria parasite population structure, HLA type, and down-regulation of the cellular immune response. Overall, the results of our study clearly indicate that immunologic nonresponsiveness has to be addressed in any vaccine development strategy involving CS and MSP-1 proteins.

A comparison between the proliferative responses among the settlement groups showed that PBMC of the miners responded more frequently to the P. falciparum CS and MSP-1 recombinant proteins than those of the farmers. Nevertheless, both groups responded equally well to PHA. The proportion of gold-miners and farmers who had their last malaria infection with this parasite. The groups were similar for this variable, i.e., four (29%) of 14 miners and 13 (34%) of 38 farmers (P > 0.05, by Fisher’s exact test) had their last infection with P. falciparum. The cellular response among these individuals corroborated a higher responsiveness among miners than farmers.

The fact that the miners experienced a higher number of previous malaria episodes than farmers supports the hypothesis that their behavioral patterns place them at a higher risk of exposure to infected mosquitoes. In this area, the gold-mining operations function with no interruption; the miners work in 12-hr shifts (day/night) in giant excavation pits, where they are exposed to mosquito bites. Moreover, the miners usually live in shelters with no lateral walls at the margin of the forest. Consequently, they are exposed to the exophilic mosquitoes even when they are resting. Open cast mining operations provide new areas of water catchment that are potential breeding sites for mosquitoes, which increase human-vector contact. Farming activities, however, are carried out only during the day, thus allowing less exposure to vectors. In this group, local epidemiologic surveillance is better since they have fixed residences in houses with lateral walls.

Unfortunately, we did not capture mosquitoes in this area. The possibility that miners and farmers were exposed to different vectors seems unlikely since they were living within the same malarial area. In many cases, the mining and farming areas are contiguous; therefore, the level of exposure to vectors is probably the most important factor that contributes to the differences in the immune response.

The correlation between a higher risk of malaria exposure, as represented by the gold-miners, and a positive cellular response does not necessarily imply causality. Confounding factors that may account for the differences in the frequency of responders are genetic background and nutritional status. However, genetic differences between gold-miners and farmers are unlikely since 1) the study subjects originated from the same regions of the country, and 2) based on skin color and accent, there appear to be no great differences between the two groups. In addition, most of the Brazilian population contains a significant amount of racial mixing.

It has been demonstrated that nutritional deficiency can suppress the cell-mediated immune response. We did not investigate caloric/protein intake; however, gold-miners and

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**Table 2**

Analysis of the proliferation responses to recombinant *Plasmodium falciparum* proteins rPfMSP-1 and rPfCS using peripheral blood mononuclear cells (PBMC) from miners and farmers with a history of clinical malaria*

| Cellular response to | Individuals with a positive stimulation index (SI) | Groups | Overall frequency of response
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<tbody>
<tr>
<td></td>
<td>Miners (n = 14)</td>
<td>Farmers (n = 38)</td>
<td>(n = 52)</td>
</tr>
<tr>
<td>rPfCS No. (%)</td>
<td>8 (57)</td>
<td>4 (10)</td>
<td>12 (23)</td>
</tr>
<tr>
<td>SI&lt;sup&gt;†&lt;/sup&gt; (mean ± SD)</td>
<td>2.9 ± 1.7</td>
<td>1.0 ± 1.5</td>
<td>t = 3.84, P &lt; 0.05</td>
</tr>
<tr>
<td>rPfMSP-1 No. (%)</td>
<td>9 (64)</td>
<td>11 (29)</td>
<td>20 (38)</td>
</tr>
<tr>
<td>SI&lt;sup&gt;†&lt;/sup&gt; (mean ± SD)</td>
<td>3.9 ± 3.2</td>
<td>2.0 ± 2.7</td>
<td>t = 2.14, P &lt; 0.05</td>
</tr>
<tr>
<td>PHA No. (%)</td>
<td>14 (100)</td>
<td>38 (100)</td>
<td>52 (100)</td>
</tr>
<tr>
<td>SI&lt;sup&gt;†&lt;/sup&gt; (mean ± SD)</td>
<td>160.0 ± 97.0</td>
<td>186.0 ± 118.0</td>
<td>t = 0.89, P &lt; 0.05</td>
</tr>
</tbody>
</table>

* rPfMSP-1 = recombinant *P. falciparum* merozoite surface protein-1; rPfCS = recombinant *P. falciparum* circumsporozoite protein; PHA = phytohemagglutinin.

† The SI was calculated as (the mean cpm of antigen cultures - mean cpm of yeast extract)/mean cpm of unstimulated cultures. The PBMC from the controls living outside the endemic area (n = 16) did not proliferate when stimulated with either recombinant protein. The mean ± SD SI values were 0.2 ± 0.25 and 0.3 ± 0.35 for rPfCS and rPfMSP-1, respectively.
farmers responded with the same intensity to the mitogen. This result suggests that possible differences in the nutritional status between the two groups is unlikely to explain the differences in their cellular response to recombinant P. falciparum proteins. In addition, if there were differences in nutritional status, the farmers would have better nutritional intake, but this group had a lower cellular response compared with the miners.

Knowledge of the natural immune response to malaria is a prerequisite for the optimal design of a vaccine. Studies carried out in various malaria-endemic areas have shown wide variations in the cellular response regarding the transmission patterns. We have demonstrated that individuals living in the same Brazilian malaria-endemic area, but with different work-related activities associated with malaria transmission, had significant differences in the frequency and magnitude of their in vitro cellular response. Although our results are limited to a small number of individuals, they underline the need for a better understanding of the relationship between immune response and the risk factors associated with work activities and life styles of the various social groups. Such studies should provide baseline information that will be crucial for understanding the effects of vaccines now in development.

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