LOW CELLULAR RESPONSE IN VITRO AMONG SUBJECTS WITH LONG-TERM EXPOSURE TO MALARIA TRANSMISSION IN BRAZILIAN ENDEMIC AREAS

ÉRIKA M. BRAGA, LUZIA H. CARVALHO, COR J. F. FONTES, AND ANTONIANA U. KRETTLI
Departamento de Parasitologia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil;
Centro de Pesquisas René Rachou, Fiocruz, Belo Horizonte, Minas Gerais, Brazil;
Departamento de Clínica Médica, Universidade Federal de Mato Grosso, Cuiabá, Mato Grosso, Brazil

Abstract. The cellular and humoral immune responses to Plasmodium falciparum and P. vivax recombinant circumsporozoite (rCS) proteins were studied in two populations in the Brazilian malaria-endemic region. One group of subjects lived in an urban area that was free from the risk of malaria but was exposed to the disease through short visits to the endemic area. The other group had lived for approximately 10 years in a rural area, where they were continuously exposed to transmission. Proliferative responses to rCS proteins were observed in 50% of 16 adults not continuously exposed to malaria but in only five of 48 subjects (10%) resident in the transmission area. The antibody responses to rCS proteins were approximately 50% in both groups. The interferon-γ (IFN-γ) response evaluated only among continuously exposed subjects was low. There was no association between the presence of antibodies and the detection of proliferative or IFN-γ T cell responses. These findings suggest that continuous exposure to malaria in areas of low endemicity may lead to a specific decrease of the in vitro T cell function.

INTRODUCTION

Protective immunity to sporozoite-induced malaria infection has been observed in experimental models and in human volunteers. A putative target antigen for protection is the circumsporozoite (CS) protein, expressed in sporozoites and in the intrahepatic stages. The CS antigen is the best characterized of the anti-sporozoite vaccine candidates, and the results of clinical trials represent a considerable advance in the development of malaria vaccines. Before field trials are performed, it is important to understand the immune mechanisms involved in natural transmission, especially where there are multiple parasite strains and variable levels of exposure to the vectors, as in Brazil.

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 subjects may experience several infections by Plasmodium falciparum or P. vivax, with clinical symptoms of variable degrees of intensity. This picture differs from that in hyperendemic areas such as equatorial Africa where malaria causes nearly three million deaths a year, mostly in children. Transmission in this African area is stable and most adults acquire protective immunity that is reflected in the rarity of clinical symptoms and low density of parasites in the peripheral blood of infected adults.

Few studies have been conducted on the cellular immune response against CS protein in subjects living in endemic areas of Brazil. In vitro tests of T cell proliferation against P. vivax CS protein showed that 32% of such subjects produced a positive response. Parallel tests of cellular responsiveness to P. falciparum and P. vivax recombinant CS (rCS) proteins, performed in areas where both parasites are transmitted, showed an overall positive rate of 45% in subjects recovering from acute malaria infection. Although approximately half the people exposed to malaria in areas where transmission is low and unstable have T cells that recognize CS epitopes, these previous studies focused on subjects exposed for short periods of time, ranging from a few months to a few years. The mass migration of settlers to gold-rich areas or governmental agricultural settlement projects in the Amazon during the 1970s meant that distinct groups are now exposed to malaria over decades. This is a new epidemiologic profile that could provide information about the protective immunity and modulation of the T cell responsiveness.

The temporal stability of peripheral T cell function and the influence of prolonged antigenic exposure on T cell response are understudied factors that may influence the human immune response in malaria. In the present study, we analyzed the T cell responses to rCS proteins and looked for possible associations between seropositivity and T cell responsiveness measured through lymphoproliferative and interferon-γ (IFN-γ) production in two different groups of subjects who reported 10 or more previous episodes of acute malaria. One group lived outside the malaria transmission area but had been exposed to the disease on several visits to the endemic region. The other group was continuously exposed to malaria transmission for a longer period, having lived for about 10 years in an endemic rural area. Significant differences in the T cell responsiveness of those two groups are described.

MATERIALS AND METHODS

Areas and Subjects. We selected individuals who had reported a high number (>10) of previous episodes of P. vivax or P. falciparum clinical malaria acquired in the state of Mato Grosso in the Brazilian Amazon endemic region. One group lived in the state capital, Cuiabá, where no malaria transmission occurs. This group consisted of 16 adults (age range = 21–50 years, median age = 31) who had become infected through short visits to mining and/or agricultural areas in the north of the state. These visits involved a total of less than a year in the endemic region. The second group consisted of 48 subjects (age range = 16–66 years, median age = 30), who had resided for 10 or more years in Terra Nova do Norte, a small rural community within the endemic region. Therefore, these individuals were continuously exposed to malaria (median time = 10.5 years). Terra Nova do Norte had a total population of 20,000 inhabitants and the Ministry of Health reported 3,090 cases of malaria in the study year of 1996. The total number of malaria cases recorded in the municipality during the previous seven years shows that the highest preva-
lence occurred in 1992, then decreased gradually (Figure 1). The basis for comparison of the Cuiabá and Terra Nova do Norte groups was that although their intensities of exposure to malaria transmission differed, both had experienced more than 10 previous episodes of acute malaria, each of which had been treated by the Ministry of Health.

Control subjects for cellular proliferation (n = 16) and for antibody assays (n = 40) consisted of healthy adult volunteers who had never been exposed to malaria transmission or visited the malaria-endemic region.

**Blood Sample Collection.** All subjects were submitted to a questionnaire that included information on past malaria and previous treatments. Consent to draw blood was obtained from each individual according to the Fundação Oswaldo Cruz Ethics Committee (Ministry of Health, November 26, 1994). Venous blood samples (20 ml per subject) were drawn in Vacutainer® (Becton Dickinson, Oxnard, CA) heparinized tubes. Giemsa-stained thick-blood smears were examined at this point to ensure that all subjects were parasite free. We elected to study only parasitologically negative convalescent individuals since the in vitro cellular response to malaria antigens is known to be immunosuppressed or diminished during acute infections. The median times of the most recent malaria infection among the subjects evaluated in Cuiabá and Terra Nova do Norte populations were 22 ± 20 and 28 ± 21 days (± SD), respectively, after the specific treatment for malaria.

**Antigens.** Two recombinant CS proteins expressed in Saccharomyces cerevisiae and transformed with plasmids containing DNA of the P. falciparum T4 isolate and P. vivax Belém isolate were produced and kindly provided by the Chiron Corporation (Emeryville, CA). These clones (rPfCS and rPvCS) contain approximately 70% of the entire CS protein, including the repeats and parts of the flanking N- and C-terminal sequences. Control antigen included an extract of S. cerevisiae (Diło, Detroit, MI) and the mitogen phytohemagglutinin (PHA; Sigma, St. Louis, MO). These were used in all lymphocyte proliferation assays.

**Antibody Measurement.** An enzyme-linked immunosorbent assay was used to detect total IgG antibodies and was performed as described. The serum dilution used was 1:40. For the recombinant antigens rPvCS and rPICS, the final optical density (OD) at 405 nm was calculated by subtracting the OD obtained with the yeast extract (antigen control). The threshold of positivity (cut-off point) was an OD value of 0.4 for rPICS and 0.1 for rPvCS, based on the mean + 2 SD of the OD obtained from the 40 healthy controls (0.16 ± 0.12 for rPICS and 0.02 ± 0.04 for rPvCS).

**In Vitro Proliferation Assay.** Mononuclear cells from heparinized peripheral blood mononuclear cells (PBMC) were isolated and used in proliferative assays in triplicate as described. Briefly, PBMC were purified in Percoll gradient (2.5 × 10⁵ cells), seeded in 96-well plates (Costar, Cambridge, MA), and the antigens (rPfCS, rPvCS, and yeast extract) were added to a final concentration of 2.5 μg/ml to each well in culture medium (RPMI 1640 medium; Sigma). This concentration was previously defined based on the reactivity of PBMC from positive subjects living in malaria-endemic areas of Brazil. Control cells were cultured with PHA (12.5 μg/ml) or with medium only. For the recombinant proteins (rPvCS or rPICS), the specific stimulation indices, expressed as SI, were calculated as the mean counts per minute (cpm) of antigen-stimulated cultures minus the mean cpm of the yeast extract control divided by the mean cpm of unstimulated cultures. A test result was considered positive if the SI > 2.0. Negative values, indicating a proliferative response to the yeast antigen higher than that to CS antigen, were plotted as zero.

**Statistical Analysis.** Pearson correlation was used to assess an association between antibody and proliferative responses to the two recombinants CS proteins used in the study. Differences in proportions were tested by the chi-square test. P values < 0.05 were considered significant.

**RESULTS**

**Antibody Response.** The antibody responses obtained for the malarial groups and the controls are summarized in Table 1. Anti-rPfCS IgG antibodies were detected in 39% of the subjects sporadically exposed to malaria (Cuiabá group) and in 42% of those continuously exposed (Terra Nova do Norte group). These values are statistically similar (P > 0.05), as were the responses of antibodies to rPvCS (50% in both groups). The mean antibody levels (absorbance) against rPfCS or rPvCS were not significantly different (P > 0.05) between the two groups (Table 1). None of the 40 healthy controls had detectable IgG antibodies to the CS antigens.

**Cellular Proliferative Response to rPfCS and rPvCS.** The distribution of the PBMC stimulation indices to recombinant CS proteins and mitogen in 64 subjects convalescing from P. vivax or P. falciparum infections is shown in Figure 2. There was a significantly lower response to the two CS proteins (rPfCS and rPvCS) in the Terra Nova do Norte group, resi-
Prevalence of IgG against two recombinant circumsporozoite (rCS) proteins of *Plasmodium falciparum* (Pf) and *P. vivax* (Pv) (rPfCS and rPvCS) in subjects exposed briefly (Cuiabá [CB]) or continuously (Terra Nova do Norte [TNN]) to malaria transmission in different endemic localities in the state of Mato Grosso, Brazil, and in controls (individuals never exposed to malaria) 

<table>
<thead>
<tr>
<th>Groups living in</th>
<th>rPfCS</th>
<th>rPvCS</th>
<th>rCS (either protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%), Mean ± SD absorbance</td>
<td>No. (%), Mean ± SD absorbance</td>
<td>No. (%), positive</td>
<td></td>
</tr>
<tr>
<td>CB (16)</td>
<td>3 (39%)*, 0.23 ± 0.20</td>
<td>8 (50%)*, 0.21 ± 0.07</td>
<td>10 (63%)*</td>
</tr>
<tr>
<td>TNN (48)</td>
<td>20 (42%)*, 0.43 ± 0.33</td>
<td>24 (50%)*, 0.13 ± 0.14</td>
<td>32 (67%)*</td>
</tr>
<tr>
<td>Non-endemic area (40)</td>
<td>0 (0%), 0.16 ± 0.12</td>
<td>0 (0%), 0.02 ± 0.04</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

* Chi-square test compared the groups in Cuiabá or in Terra Nova do Norte. $P > 0.05$ for a comparison of both groups for each antigen.

LOW CELLULAR RESPONSE AFTER LONG EXPOSURE TO MALARIA

We describe here different patterns of cellular immune response between two groups of subjects exposed to endemic malaria transmission in Brazil and who had experienced at least 10 previous malaria episodes. The proliferative T cell response to malaria rCS proteins was high ($\geq$ 50%) in subjects living outside the endemic region (Cuiabá group) and who had acquired the disease through visits to malarial endemic areas. In contrast, the proliferative cell response was poor ($\leq$ 10%) in the group of long-term residence in a rural community within the endemic area (Terra Nova do Norte group).

A strong proliferative T cell response to malarial antigens during the convalescent phase of infection has been previously shown in African children.\textsuperscript{11,15} Cells from adults who reported less than five years of malaria exposure in Brazilian endemic areas also had a significant proliferative response to CS proteins.\textsuperscript{7,8,16} The high frequency of proliferative response now observed in the CB group is consistent with those earlier studies. However, a low level of *in vitro* cellular proliferative response to CS proteins in subjects continuously exposed to *P. falciparum* and *P. vivax* transmission for at least 10 years (Terra Nova do Norte group) is reported for the first time in Brazil. We interpret this as a result of possible acquisition of protective immunity, described in other regions of the world, for instance, in Asia.\textsuperscript{17} This study, conducted in Sri Lanka, showed a low malaria-specific T cell proliferative response in adults who were recovering from acute *P. vivax* malaria and who experienced repeated infections during a life-long exposure to seasonal transmission.

Malaria-specific proliferative T cell responses to various malaria antigens are commonly observed to be higher in non-immune or semi-immune rather than in the immune subjects. It thus appears that T cell responses to certain antigens are actively down-regulated as a function of exposure to parasites.\textsuperscript{18} Further support for this concept can be found in our study, reporting markedly lower CS protein-specific T cell proliferative response in individuals continuously exposed to transmission compared to briefly exposed individuals. However, a longitudinal study would be required to determine whether these low responders are less susceptible to infection than high responders.

Multi-parameter analyses have shown that single-parameter analysis underestimates T cell recognition of peptide antigens.\textsuperscript{18} Future studies should investigate other parameters that measure the cellular response, such as other cytokine
levels than IFN-γ in the culture supernatants of PBMC stimulated with defined antigens.

When one considers that all the subjects studied were convalescent and not parasitemic at the time of blood sample collection, sequestration of responding T cells in lymphoid tissues is unlikely to explain the poor cellular responses detected in the Terra Nova do Norte group. However, even in the absence of clinical disease, the presence of low level parasitemia can affect the ability of peripheral T cells to respond.19,20 Indeed, this may be the case in the subjects now reported who had undetectable parasitemia when analyzed by conventional Giemsa-stained blood smears.

It has been assumed that protection against pre-erythrocytic stage malaria is mediated by CD8+ cytotoxic T lymphocytes and that cytokines such as IFN-γ have also been implicated. It has also been shown that IFN-γ induces the production of nitric oxide in vitro and in vivo following infection with P. berghei, P. yoelli, or P. falciparum sporozoites.21 In addition, acquisition of clinical immunity to malaria has been associated with a decreased capacity to produce IFN-γ in African adults in response to P. falciparum antigens, compared with non-immune Europeans who had a single case or a few cases of acute malaria.22 Thus, continuous exposure to malaria-infected mosquitoes in hyperendemic areas may lead to decreased regulation of the in vitro T cell function against malaria antigens. We also observed that the levels of IFN-γ in response to CS proteins were low among individuals continuously exposed in Terra Nova do Norte, an area of low malaria endemicity, thus providing further evidence of decreased T cell regulation (Braga EM and others, unpublished data). We suggest that long-term exposure to hyperendemic, unstable malaria transmission, such as in Brazil, may also downregulate the in vitro cellular response against sporozoite antigens and seems to be a new phenomenon in Brazil. We interpret this data as a possible result of acquisition of partial immunity. However, further investigations are required to verify whether these observations are applicable in different epidemiologic settings, objective of our ongoing work.

The mechanism responsible for the poor T cell response in individuals exposed for longer periods to antigenic activation is uncertain. A permanent exposure of the immune system to plasmodial antigens might induce increased levels of mononuclear cell apoptosis that causes a deletion of reactive T cells, as described for superantigens.23,24 This resulted in less PBMC proliferation in response to specific antigenic pressure in vitro. In human malaria, the percentage of cell mortality by apoptosis is significantly higher in lymphocytes from subjects living in areas of holoendemic malaria transmission than in those from individuals who are less exposed to the disease.25,26

B and T cell allelic diversities to the CS protein have been documented in human malaria parasites.27 It has been assumed that T cells primed by a determined epitope variant will not generally respond to another variant of the same epitope. It is therefore possible that the P. vivax and P. falciparum isolates present in the Terra Nova do Norte region differ from those used to produce the rCS proteins used in our study, thus contributing to the poor cellular response detected in the subjects from this group.

The possibility of major histocompatibility complex (MHC) restriction seems an unlikely explanation for the low responsiveness observed since the Brazilian population is genetically heterogeneous due to mixing of immigrant groups. In addition, the universal T cell epitopes capable of binding to multiple MHC class II molecules seem to be included in the rCS C-terminal region of both P. vivax and P. falciparum.28,29

Although cellular responses were low in subjects from Terra Nova do Norte, their antibody responses were frequent, high, and similar to those of the Cuiabá group. Previous studies in Papua New Guinea30 and in Brazil7 demonstrated that the proliferative response against CS antigens did not correlate with the corresponding antibody levels. Limited CD4+ T cell responses were observed in residents of Papua New Guinea naturally exposed to the CS protein.18 The data suggest that although proliferative memory T cells are driven into an anergic state by chronic antigenic exposure, they may maintain the ability to help B cells, a hypothesis corroborated by our present data. It is possible that among low-responding individuals continuously exposed to malaria transmission T cell-independent mechanisms predominate.

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Authors’ addresses: 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; and Antoniana U. Krettli, Laboratório de Malária, Centro de Pesquisas René Rachou/Fiocruz, Av. Augusto de Lima 1715, Barro Preto CP1743, 30190-002, Belo Horizonte, Minas Gerais, Brazil. Cor J. F. Fontes, Departamento de Clínica Médica, Hospital Júlio Müller, Universidade Federal de Mato Grosso, Rua L S/N, Jardim Alvorada, 78070-150, Cuiabá, Mato Grosso, Brazil.

Reprint requests: Antoniana U. Krettli, Laboratório de Malária, Centro de Pesquisas René Rachou/Fiocruz, Av. Augusto de Lima 1715, Barro Preto CP1743, 30190-002, Belo Horizonte, Minas Gerais, Brazil.

Telephone: 55-31-3295-3566, Fax: 55-31-3295-3115, E-mail: akrettli@epgpr.fiocruz.br.

REFERENCES


