ANTIBODIES TO PLASMODIUM VIVAX APICAL MEMBRANE ANTIGEN 1: PERSISTENCE AND CORRELATION WITH MALARIA TRANSMISSION INTENSITY

CRISTIANE G. MORAIS, IRENE S. SOARES, LUZIA H. CARVALHO, COR J. F. FONTES, ANTONIANA U. KRETTLI, AND ÉRIKA M. BRAGA*

Departamento de Parasitologia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil; Departamento de Análises Clínicas e Toxicológicas, Universidade de Sao Paulo, Sao Paulo, Brazil; Universidade Federal de Mato Grosso, Cuiabá, Mato Grosso, Brazil; Centro de Pesquisas René Rachou/FIOCRUZ, Belo Horizonte, Minas Gerais, Brazil

Abstract. The antibody responses to the apical membrane antigen 1 of the Plasmodium vivax (PvAMA-1) were investigated in subjects living in areas of Brazil with different levels of malaria transmission. The prevalence and the levels of IgG to PvAMA-1 increased with the time of exposure. The frequency of a positive response and the mean IgG level were higher in areas where malaria prevalence was more intense, especially among non-infected subjects exposed to moderate transmission over a period of 20 years. The proportions and levels of IgG1 and IgG3 isotypes were significantly higher among those subjects with long-term exposure. Antibodies, mainly IgG1, to PvAMA-1 persisted for seven years among subjects briefly exposed to malaria in an outbreak outside the Brazilian malaria-endemic area. These data show the highly immunogenic properties of PvAMA-1 and emphasize its possible use as a malaria vaccine candidate.

INTRODUCTION

Antigens of Plasmodium located on the surface or in the apical organelles of merozoites have been characterized as targets for protection or as possible vaccines against malaria. Among several vaccine candidates, apical membrane antigen 1 (AMA-1), an asexual blood stage antigen, is considered to be an important candidate malaria vaccine antigen.1 This protein is present in all Plasmodium species examined, as well as in other Apicomplexa.2–4 This antigen is a type I integral membrane protein and in all characterized Plasmodium vivax AMA-1 (PvAMA-1) genes 16 invariant Cys residues are encoded in the ectoplasmic region; analysis of the disulfide bond pattern suggests a division into three distinct domains.5 Analysis of the crystal structure of the ectoplasmic region of the PvAMA-1 confirmed the division of this region into three structural domains.6 Comparison of the PvAMA-1 structure with other known three-dimensional structures showed that domains I and II are structurally similar to each other and belong to the PAN module superfamily.6 This superfamily is related to proteins with diverse adhesion functions binding to protein or carbohydrates receptors.7

AMA-1 is synthesized late during the development of parasite schizonts as a precursor of an 83-kD protein that is initially located in the micronemes of the apical complex of merozoites and sporozoites.7 It is processed to a 66-kD form before its relocation into the surface of merozoites.7 Once on the surface, this form is redistributed and undergoes two C-terminal cleavages, giving rise to 48-kD and 44-kD soluble forms.8–11 The function of AMA-1 is not well understood, but its stage specificity and location suggest that this protein is involved in the process of invasion of host erythrocytes.12,13

The inclusion of AMA-1 as malaria vaccine candidate is supported by evidence from studies of malaria in animal models.14,15 Furthermore, in malaria-endemic African populations, a considerable proportion of individuals has naturally acquired antibodies and T cell reactivities to AMA-1.16–18

In spite of the critical importance of the AMA-1, few seroepidemiologic studies have been conducted to understand the dynamics of naturally acquired humoral immune responses to this molecule in populations exposed to different patterns of malaria transmission. Most of those studies have been conducted in holo-hyperendemic areas and P. falciparum AMA-1 has been used as a target for antibody detection.16–18

Although P. vivax causes less mortality than P. falciparum, it has an enormous socioeconomic impact, particularly in South America and Asia. In Brazil, P. vivax was responsible for approximately 80% of the 459,013 cases of malaria reported in 2004 (Brazilian Ministry of Health). Given the critical importance of understanding naturally acquired immunity to P. vivax antigens, we conducted an immunoepidemiologic study focusing on the antibody response to PvAMA-1 in distinct areas within the Brazilian Amazon malaria-endemic region. We also evaluated the persistence of specific antibodies to PvAMA-1 among subjects briefly exposed to P. vivax transmission outside the malaria-endemic area.

MATERIALS AND METHODS

Study areas, subjects, and blood sample collection. We analyzed four different groups of non-infected adults who had been exposed to malaria transmission in the Brazilian Amazon area (Table 1). A complete health questionnaire was applied to all study participants. Ethical clearance for this study was obtained from the institutional review board of the Federal University of Minas Gerais, Brazil (April 15, 1998; #007/98 and May 16, 2001; #096/01). All participants reported at least one malaria infection diagnosed by microscopy; the date and species involved in this most recent episode were recorded. Since most subjects were migrants from areas in Brazil not endemic for malaria, their ages do not correlate with cumulative exposure to malaria. Past malaria exposure was therefore estimated as the length of residence or exposure to malaria in malaria-endemic areas in Brazil. These parameters were used to classify the four groups.

The first group lived in Belém, the capital of Pará State. It was composed of 59 persons (median age = 29 years) who had acquired P. vivax malaria after a brief exposure (a few
The concentration of PvAMA-1 into Vacutainer® heparinized tubes (Becton Dickinson, Oxford, CA). Giemsa-stained thick-blood smears were examined at this point. Control subjects consisted of 40 healthy adult volunteers living in Belo Horizonte in Minas Gerais State who had never been exposed to the malaria transmission or visited the malaria-endemic region.

**Antigens.** Recombinant PvAMA-1 contains the amino acid sequence (43–487) of the ectodomain of the *P. vivax* BEL-12 isolate.\(^23\) This protein was expressed in *Escherichia coli* and purified as described previously.\(^23\)

**Antibody measurement.** An enzyme-linked immunosorbent assay (ELISA) for total IgG antibodies was performed as previously described.\(^22\) The concentration of PvAMA-1 used was 1.0 μg/mL. All samples were diluted 1:100 and evaluated for total IgG using peroxidase-conjugated anti-human IgG antibodies (Sigma, St. Louis, MO).

The ELISA to detect IgG subclasses was performed as previously described.\(^22\) The serum dilution used was 1:50 and mouse monoclonal antibodies to human 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) diluted according to the manufacturer’s specifications. Monoclonal antibody binding was detected with peroxidase-conjugated anti-mouse immunoglobulin (Sigma).

The threshold of positivity (cut-off value) was obtained by testing 40 different negative control sera from individuals not exposed to malaria from Belo Horizonte. The mean optical density value at 490 nm (OD\(_{490}\)) ± 3 SD for duplicate determinations in negative sera was used as the cut-off value for different subclasses. The thresholds of positivity were 0.32 for IgG, 0.15 for IgG1, 0.13 for IgG2, 0.1 for IgG3, and 0.06 for IgG4.

The reactivity index (RI) was obtained to compare the levels of different subclasses (IgG or IgG isotype) in the same subject or among distinct groups. The RI value was calculated by dividing the mean OD value for each test sample assayed by the cut-off value for each subclass tested using sera from healthy individuals (control group). Samples with an RI > 1 were considered positive.

**Statistical analysis.** Data were analyzed using Epi-Info version 6.03 (Centers for Disease Control and Prevention, Atlanta, GA). The chi-square test was used to compare proportions and the Kruskal-Wallis test was used to analyze the significance of differences between group values. Significance was set at the 5% level.

**RESULTS**

**Relationship of IgG antibodies to PvAMA-1 and malaria exposure.** The proportions of PvAMA-1 IgG-positive subjects increased with exposure to malaria transmission (\(P < \))
It reached a peak of 95% in those subjects with long-term exposure in the Brazilian malaria-endemic area (Apiacás group). However, similar proportions of IgG antibodies to PvAMA-1 were detected in subjects in the Belém (59%), Cuiabá (71%), and TNN (71%) groups. Persons in the Belém group, which was composed of subjects who reported a single P. vivax infection, had a high IgG antibody response to PvAMA-1 (59%).

The levels of IgG antibodies to PvAMA-1 (measured as RIs) are shown in Figure 1. High levels of specific IgG (RI ≥ 5) were observed among 36% of the subjects living in Apiacás with long-term exposure to malaria. The IgG-positive responses ranged to 3% to 9% in the other groups \((P < 0.001)\). Proportions and levels of PvAMA-1-specific IgG1 and IgG3 also showed differences related to exposure, being highest for individuals living in Apiacás \((P < 0.05)\) (Figure 2A and B).

**Relationship between most recent malaria episode and antibodies to PvAMA-1.** Since subjects from Cuiabá, TNN, and Apiacás had experienced various numbers of episodes of malaria caused by *P. vivax* and/or *P. falciparum*, we analyzed the effect of the most recent malaria episode on the antibody responses against PvAMA-1. As shown in Figure 3A, individuals living in Cuiabá whose most recent infection was with *P. vivax* showed higher proportions of IgG- and IgG1-positive responses when compared with those whose most recent malaria episode was caused by *P. falciparum*. In TNN, the results showed a non-significant tendency towards increased prevalence of antibodies to PvAMA-1 among individuals whose most recent malaria episode was caused by *P. vivax* \((P > 0.05)\) (Figure 3B). Sera from subjects in the Cuiabá group whose most recent infection was with *P. vivax* showed higher levels of IgG when compared with those whose most recent infection was with *P. falciparum*.

**Longevity of IgG antibodies to PvAMA-1.** Twenty-five persons living in a rural community in Minas Gerais who were briefly exposed to a *P. vivax* malaria outbreak outside the malaria-endemic area were evaluated to measure the persistence of specific antibodies. Sera were tested in parallel in all samples obtained at two different time points. IgG antibodies to PvAMA-1 were detected in 43% of the subjects who had had malarial symptoms eight months after transmission (Figure 4). Among the prophylactically treated individuals, 64% were also positive for IgG antibodies to PvAMA-1. Seven years later, positive responses were still detected in approximately 36% of both groups. Specific IgG1 was the predominant subclass, regardless of malaria symptoms. It was first detected in 13 individuals (52%) and was still present in nine (36%) persons after seven years. Similar distributions of IgG or IgG1 antibodies to PvAMA were found in symptomatic and asymptomatic subjects evaluated at the two time points.

**DISCUSSION**

Although AMA-1 is an important vaccine candidate antigen, there have been few serologic studies on the nature of immune responses to this protein in persons living in malaria-endemic areas. Previous studies have demonstrated the immunogenicity of *P. falciparum* AMA-1 among African subjects.\(^{10–18}\) Currently available seroepidemiologic data on the
The homologous protein is limited to a study in Brazil that showed a significant frequency of positive antibody responses to PvAMA-1 (85%) among individuals with patent *P. vivax* infection. However, no studies have addressed the effect of the intensity of malaria exposure on the development of antibodies to PvAMA-1.

We have demonstrated that a recombinant protein containing the ectodomain of *P. vivax* AMA-1 is naturally immunogenic in Brazilian persons who had distinct degrees of malaria exposure. The high prevalence of PvAMA-1-specific antibodies could be explained by the fact that the region coding the variable domain of the PvAMA-1 gene shows limited polymorphism among Brazilian isolates.

Since the IgG subclasses produced in response to a given antigen may determine the function of the antibody, we determined the proportions and levels of IgG isotypes of PvAMA-1. Analyses of IgG subclasses responses showed that the prevalence and the levels of PvAMA-1-specific IgG1 and IgG3 were higher among subjects with long-term exposure to malaria. It is known that IgG1 and IgG3 isotypes have been implicated in antibody-mediated protective immunity against *Plasmodium* blood stages. Thus, the high level of cytotoxic antibodies among subjects who were continuously exposed to malaria for more than 20 years without detectable parasitemias may be the result of acquisition of partial immunity against malaria, as previously suggested.

High proportion and levels of IgG3 were detected in miners living in Apiacás who were not infected with *Plasmodium*. It is important to note that at the time of blood collection, those subjects were negative for *Plasmodium* by conventional microscopic examination of thick blood smears and a nested polymerase chain reaction for the 18S small subunit ribosomal rRNA gene. Since IgG3 has a relatively short half-life, the antibody response to PvAMA-1 for this isotype may be caused by frequent re-exposure to the parasite, which is required to increase levels of these antibodies.

Long-term antibody production is one hallmark of effective vaccination and is an important characteristic of immunologic memory. B cell memory seems to depend on the persistence of stimulating antigen maintained over long periods by im-
mune complex on follicular dendritic cells of germinal centers in secondary lymphoid organs, or an antigen-independent mechanism such as the limitation of the number of long-lived plasma cells that allows the immune system to maintain a stable humoral immunologic memory over long periods.\textsuperscript{30} We have shown that that humoral immunity to \textit{P. vivax} can be maintained even in the absence of continuous re-exposure to the parasite.\textsuperscript{21–23} Based on this finding, in the present study we also determined the IgG antibody response against \textit{PvAMA-1} in subjects exposed to a previous outbreak of \textit{P. vivax} malaria outside the malaria-endemic Amazon region. Our results showed that eight months after transmission IgG antibodies to \textit{PvAMA-1} were present in six subjects who had had malarial symptoms during the outbreak and persisted among five of them. These results are consistent with a one-year longitudinal study in Kenya in which IgG antibody responses to \textit{P. falciparum} AMA-1 were persistent.\textsuperscript{16} Interestingly, eight months after focal malaria transmission, the prevalence of antibodies to \textit{PvAMA-1} was also higher (64\%) among the asymptomatic prophylactically treated subjects. However, similar distributions of IgG or IgG1 antibodies to \textit{PvAMA-1} were found in both groups regardless of malaria symptoms.

Since AMA-1 is also localized in the micronemes of the apical complex of sporozoites, it may contribute to the presence of antibodies to \textit{PvAMA-1} among those prophylactically treated subjects that could abolish the infection before detection of the parasites by thick blood smears. Our previous study conducted in the same area using a circumsporozoite protein of \textit{P. vivax} showed a persistent specific antibody response in the absence of reinfection, regardless of malaria symptoms.\textsuperscript{21} These data contrast with our previous data for the same population focusing in blood-stage antigens (C-terminal of \textit{P. vivax} merozoite surface protein 1)\textsuperscript{21,22} which showed that long-term specific antibodies were persistent only among subjects who had clinical malarial symptoms during the malaria outbreak.

In conclusion, we have found that proportions and levels of antibodies against \textit{PvAMA-1} are correlated with the intensity of exposure to malaria in areas with low-to-moderate transmission in Brazil. These observations of long-lasting specific IgG1 indicate the high immunogenicity of \textit{PvAMA-1} and emphasize its use as a possible \textit{P. vivax} malaria vaccine.

Received June 14, 2005. Accepted for publication December 1, 2005.

Financial support: This study was supported by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (grant no. CBB3232/97).

Authors' addresses: Cristiane G. Morais and Érika M. Braga, Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, 31270-901, Belo Horizonte, Minas Gerais, Brazil. Irene S. Soares, Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Prof. Lineu Prestes 580, Bloco 17, 05508-900, Sao Paulo, Brazil. Luzia H. Carvalho and Antonia U. Krettli, Laboratório de Malária, Centro de Pesquisas René Rachou/FIOCRUZ, Av. Augusto de Lima 1715, Barro Preto, 30190-002, Belo Horizonte, Minas Gerais, Brazil. Cor Jesus 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: Érika M. Braga, Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, 31279-901 Belo Horizonte, Minas Gerais, Brazil, Telephone: 55-31-3499-2876, Fax: 55-31-3499-2970, E-mail: embraga@ich.ufmg.br.

REFERENCES


