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**Differential Antibody Responses to *P. falciparum* Glycosylphosphatidylinositol Anchors
in Patients With Cerebral and Mild Malaria**

Running title: IgG to GPI and cerebral malaria

Ronald Perraut,^{a*} Bacary Diatta,^b Olivier Garraud,^c Ronan Jambou,^a Shirley Longacre,^d
Gowdahalli Krishnegowda,^e Alioune Dieye,^a D. Channe Gowda,^e

^aUnité d'Immunologie, Institut Pasteur de Dakar

^bHôpital Principal, Service de Réanimation, Dakar, Senegal

^cGIMAP – EA 3064, Faculty of Medicine, University of Saint-Etienne, France

^dUnité d'Immunologie Moléculaire des Parasites, CNRS URA 1960, Institut Pasteur, Paris,
France

^eDepartment of Biochemistry and Molecular Biology, Pennsylvania State University College
of Medicine, Hershey, PA, USA.

*Address for correspondence: Dr Ronald PERRAUT, Unité d'Immunologie, Institut Pasteur
de Dakar, 36 Av. Pasteur, BP-220, DAKAR, SENEGAL - Phone: 221 839 92 45 FAX: 221
839 92 10 - Electronic Mail: perraut@pasteur.sn.

Abstract

Glycosylphosphatidylinositol (GPI) membrane anchors of *Plasmodium falciparum* surface proteins are thought to be important factors contributing to malaria pathogenesis, and anti-GPI antibodies have been suggested to provide protection by neutralizing the toxic activity of GPIs. In this study, IgG responses against *P. falciparum* GPIs and a baculovirus recombinant MSP1p19 antigen were evaluated in two distinct groups of 70 patients each, who were hospitalized with malaria. In both groups, anti-GPI IgGs were found to be significantly lower in patients with confirmed cerebral malaria compared to those with mild malaria, or immune asymptomatic individuals ($P < 0.01$). In contrast, a particular marker of the anti-parasite immunity, as monitored by the anti-MSP1p19 IgG response, was similar in all categories of individuals, although significantly lower in the subgroup having a fatal outcome to cerebral malaria. These results are consistent with a potential anti-toxin role for anti-GPI antibodies associated with protection against cerebral malaria.

KEY-WORDS: *Plasmodium falciparum*; glycosylphosphatidylinositol anchors; MSP1p19; antibody response; cerebral malaria; protection.

1. Introduction

Many clinical manifestations of *P. falciparum* infection are caused by a complex cascade of events triggered during schizont rupture. Glycosylphosphatidylinositol (GPI) anchor structures are considered to be important parasite toxin candidates that could contribute to immunopathological events leading to the development of severe malaria. The patho-physiological effects of parasite GPIs have been attributed to their ability to induce the production of proinflammatory cytokines in the host, including tumor necrosis factor (TNF- α), interleukin-1 (IL1), nitric oxide (NO), and interferon (IFN- γ) [1-4]. Additionally, *Plasmodium* GPIs have been proposed to mediate hypoglycemia by mimicking the activity of insulin [5].

GPIs are ubiquitous in eukaryotes, and are primarily involved in anchoring certain cell surface proteins to plasma membranes. Compared to animal cells, GPIs are abundantly expressed in various parasite species including *Plasmodium*, *Trypanosoma*, and *Leishmania*, and these organisms contain large pools of free GPIs that are not attached to proteins [6]. Unlike protein anchored forms, free GPIs are not masked on cell surfaces, and therefore are more accessible for triggering innate immune responses [7], including pro-inflammatory cytokine secretion [8]. Although the physiological functions of the GPIs remain poorly understood, it appears likely that the parasites use GPI bioactivity to stimulate host immune responses for their own benefits. However, uncontrolled stimulation of the innate immune system is deleterious to host, and that can lead to severe clinical symptoms [7].

Since individuals living in areas of high malaria transmission have acquired immunity to malaria pathogenesis, anti-GPI antibodies have been proposed as mediators of malaria “anti-disease” immunity. Several studies have addressed the question of the protective role of anti-GPI antibodies [8-11]. In malaria endemic areas, anti-GPI IgG is produced in an age-dependent manner, correlating with the cumulative age-related acquisition of protective

immunity to malaria. It has also been observed that antibodies to GPIs are predominantly of the IgG3 subclass and are rapidly boosted in response to infection, but they are short lived [9-12]. While a recent study has shown that anti-GPI antibodies are significantly higher in children with asymptomatic infections compared to those exhibiting clinical symptoms [9, 10, 13], the observed differences were not statistically significant and there is clearly a need for more controlled studies.

In this cross-sectional study, we have investigated the potential protective role of *P. falciparum* anti-GPI IgG responses in malaria pathogenesis, in individuals from an urban area presenting at two hospitals in Dakar, Senegal with symptoms of cerebral malaria. Two sets of distinct, well-defined groups of patients with confirmed cerebral malaria were recruited in two consecutive timeframes. In parallel we measured the levels of IgG specific for baculovirus recombinant MSP1p19, the conserved 19 kDa C-terminal fragment of the 200-kDa major merozoite surface protein 1, which is anchored to the parasite surface by a GPI moiety. During merozoite invasion of erythrocytes, a major portion of N-terminal MSP1 is proteolytically cleaved, leaving MSP1p19 and its GPI anchor intact that are carried into newly invaded erythrocytes [14]. Antibody responses to PfMSP1p19 have been extensively studied and shown to be associated with clinical immunity in children and adults [15, 16]. MSP1p19 is known to induce an effective *P. falciparum* anti-parasite immune response by inducing antibodies that interfere with the merozoite invasion process [17]. The baculovirus expressed MSP1p19 antigen used in this study [18] is strongly recognized by the sera of infected individuals, and IgG responses were shown to be significantly associated with delayed infection following drug cure [19], and with clinical protection [20]. Our data show that, in both study groups, patients with cerebral malaria had significantly lower levels of anti-GPI antibodies compared to individuals with mild or asymptomatic malaria.

2. Materials and methods

2.1. Sample collection and study population

Subjects were patients living in the hypoendemic urban area of Dakar who were treated at Principal and LeDantec Hospitals, Dakar. In Dakar, over 2 million people seasonally receive an average of 0.5 infective bite per individual per year, with highly variable densities of vector anopheline mosquitoes [21]. A mean incidence of 2.4% of clinical accesses (26 cases out of 1067) were observed [13]. Blood samples collected for biological investigations from patients hospitalized for acute symptoms of malaria at different periods of time during and after the transmission season (September to December) were used. Controls consisted of samples from individuals living in Ndiop, a mesoendemic area of transmission, who are resistant to malaria pathogenesis, and were either asymptomatic or presented only mild symptoms such as transient fever and low parasitemia throughout a longitudinal follow-up carried out for several years [22]. In the Ndiop project, the longitudinal follow-up protocol was renewed yearly by assembling the villagers, and informed consent was obtained from all participants, their parents or guardians. The protocols were approved by the Ethics Committee of the Ministry of Health of Senegal.

The first study-group consisted of 70 hospitalized “adults” (≥ 13 years old, and not treated in the pediatric intensive care facilities). In this group, 35 patients had confirmed cerebral malaria and recovered with no sequelae (mean age 28 years, sampled in 1998-1999), and 35 adults hospitalized for “mild” malaria (mean age 31.7 years, sampled in November-December 1999). The immune controls for this study-group consisted of 35 adults and children aged 6 to 70 years (mean age 24.5 years) from Ndiop. These individuals were sampled in 1998 following the transmission season (with a cumulated entomological inoculation rate of 5.3 infective bites per individual). In these three groups, there was no significant difference in the age distribution.

The second study group consisted of 70 patients hospitalized with cerebral malaria, sampled from November 2000 to December 2001. Of these, 24 were aged 13 to 63 years (mean age 31 years), 28 were 2 to 12 years old (mean age 6.8 years) with confirmed cerebral malaria but recovered, and 18 adults and children 2.6 to 63 years old had a fatal outcome to cerebral malaria (mean 20.6 years). The controls for this group were: i) 63 matched individuals from Ndiop sampled in November 2001, including adults and children from 4 to 65 years (mean 19.4 years) with a cumulative entomological inoculation rate of 80 infective bites; ii) 30 individuals treated for “mild” malaria at the health centre of Dakar (6 to 46 years, mean 17.3 years); iii) 47 uninfected individuals (2 to 62 years, mean 20.6 years) living in Dakar, sampled in the context of routine biological analyses carried out at the hospital during the transmission period. In these groups, there was no significant difference in the age distribution.

After collection of blood samples, red blood cells were separated by centrifugation, and plasma stored at -20°C until used. Each set of samples has been grouped and analyzed separately.

2.2. Antigens and ELISA procedure

GPIs were isolated and purified by HPLC as described [9]. Recombinant MSP1p19 (Palo Alto allele), was produced in *Trichoplusia ni* insect cells (High Five, Invitrogen) infected with recombinant baculovirus and purified by metalloaffinity chromatography [23]. GPIs were dissolved in methanol and 50 µl per well (2 ng GPI) was transferred to flat-bottomed Immulon-4 ninety six-well microtiter plates (Dynatech, Springfield, VA). Plates were dried at 37°C and blocked with Phosphate-buffered saline containing 5% BSA (PBS-BSA). Microtiter plates were coated with 100 µl per well of MSP1p19 at a concentration of 0.5 µg/ml. Plasma samples were diluted 1:100 in PBS with 1% BSA /0.05% Tween 20, and ELISAs were performed as described previously [9, 19, 24].

The results are expressed as OD ratios, i.e., $OD_{\text{sample}}/OD_{\text{negative control}}$. The negative control was pooled plasma from Europeans not exposed to malaria. Positive responders were individuals with OD ratios > 2 (approximate mean OD + 2 SD of naive controls) [19, 24]. For positive controls, ODs were ~ 0.45 (OD ratio of ~ 6) and ~ 1.6 (OD ratio of ~ 11) for GPI and MSP1p19, respectively.

2.3. Statistical analysis

Comparisons of antibody levels between different groups were done by the Mann-Whitney rank test for non-normally distributed unpaired data. The Wilcoxon signed rank test and the Spearman rank correlation test was used for paired data. The exact Fisher's test was used to compare between groups (χ^2). P values < 0.05 were considered significant. Statistical analyses were performed using Statview 5.0[®] software (SAS Institute, Cary, NJ).

3. Results

3.1. IgG responses to GPI and MSP1p19 in the first study group

As summarized in Table 1, MSP1p19 was strongly recognized by all patients (94-100% responders) with an OD ratio > 2 . As shown in Fig. 1a, there were similar levels of MSP1p19 specific IgG in all groups, including those with asymptomatic (AM, 9.1 ± 4 OD ratio), mild (MM, 7.7 ± 2.1), or cerebral malaria (CM, 7.2 ± 2.5). In contrast, as shown on Fig. 1b, the anti-GPI IgG levels were significantly lower in individuals with CM (1.7 ± 0.8) compared to those exhibiting AM and MM (3.2 ± 3 ; 2.6 ± 3.3 OD ratio, respectively) ($P < 0.01$). The prevalence of responders was significantly lower in CM compared with AM (global test: $\chi^2 = 5.51$; $P = 0.02$). Interestingly, differences in serum antibody levels between MM and CM

groups was detectable only for GPIs but not for several *P. falciparum* recombinant protein antigens including MSP1p19 (Fig. 1a, and data not shown).

We found a significant correlation between IgG responses against MSP1p19 and GPI only in malaria-protected individuals ($P = 0.08$, $Rho = 0.47$). The levels of IgGs against GPIs and MSP1p19 were age-dependent only in Ndiop villagers ($P = 0.01$, $rho = 0.38$ and $P < 0.001$, $Rho = 0.64$, respectively). This is in agreement with the results of previous studies showing that the anti-GPI IgG responses increased with age, along with cumulative immune responses in individuals continuously exposed to *P. falciparum* [11].

3.2. IgG responses to GPI and MSP1p19 in the second study group

As in the first group, MSP1p19 was strongly recognized by all categories of individuals (71-87% positive responders), except by the urban negative controls (Table 1). However, there were no significant differences in anti-MSP1p19 antibody levels or prevalence in patients with cerebral malaria compared to those with mild malaria or immune individuals living in Ndiop, although all were significantly higher than uninfected individuals from an urban setting (figure 2a, $P < 0.001$). In contrast, anti-GPI IgG levels were significantly different in these three categories of patients; low levels in urban uninfected controls to high levels in malaria-protected individuals. Patients with cerebral malaria had slightly higher levels of anti-GPI IgGs compared to controls ($P = 0.008$), but significantly lower than in Ndiop villagers ($P = 0.0004$) (Fig. 2b). AM and MM sera showed similar prevalence and levels of IgG responses to GPI. Of note, anti-GPI IgG levels in CM tended to be lower than for MM, but were not statistically significant, in contrast to the incidence of responders, which was significantly lower in CM compared to MM (global test: $\chi^2 = 4.76$; $P = 0.03$). This intermediary result observed with the MM group was probably related to the limited number of patients.

In addition, as observed in the first study group, similar relationships between IgG responses against GPIs, MSP1p19 and age were found. As shown in Fig. 3, anti-GPI and anti-MSP1p19 antibody responses and age increased co-linearly in Ndiop villagers, exhibiting age-dependent cumulative immune responses ($P < 0.001$, Rho around 0.44), but not in patients suffering from cerebral malaria.

Importantly, in the group of hospitalized individuals, a comparison of IgG responses specific for MSP1p19 (figure 2c) or GPI (figure 2d) in patients who recovered from cerebral malaria versus those who had fatal outcomes indicated that the anti-GPI IgG levels were not substantially different. However, in fatal cases, there was a significantly lower level of anti-MSP1p19 antibody responses (mean OD ratio of 4.7) for the 50% of positive responders ($P = 0.012$ and global test: $\chi^2 = 5.45$; $P = 0.02$).

4. Discussion

In this study, we have analyzed anti-GPI IgG responses in individuals living in an urban area of low endemicity, who developed cerebral malaria following *P. falciparum* infection. Two groups of patients (CM and AM), considered to be non-immune or partially immune, were sampled from the same location during different time periods, and categorized on the basis of the clinical outcomes. These individuals, regardless of age and exposure to infective bites, were at risk for clinical episodes, and shared similar observable clinical outcomes of cerebral malaria, well documented and sharing an adequate follow-up and treatments in the intensive care unit of Dakar's hospitals. Recruitment was limited to the transmission season and restricted by the capacity of the intensive care facilities, requiring a cumulative enrollment during two consecutive seasons to collect an adequate number of comparable samples. Indeed, there was a substantial heterogeneity in such recruitment, as the individual medical care and

history of infection is not totally documented before hospitalization. In this line we conducted two independent studies.

A second category of individuals living in the mesoendemic village of Ndiop, who had acquired natural immunity (AM) in an age-dependent manner by repeated exposure, was included for comparison with CM and MM groups. Children from Ndiop were known to have a substantially higher degree of anti-malarial immunity than older adults living in Dakar (24, 26). Previous data from longitudinal studies showed similar antibody responses against conserved antigens, including MSP1, PfEMP3 and Pf332, when measured during consecutive years, at similar time periods (before or after the transmission season). Thus, such well-defined samples were considered to be relevant "immune" controls for antibody responses to the antigens investigated in this study, despite the different geographical settings (1, 12, 14-16).

The data consistently showed that, in both study groups, individuals who developed cerebral malaria had markedly lower levels of anti-GPI antibodies compared to those who had asymptomatic infections or mild symptoms. This suggests that cerebral malaria outcomes is associated with low levels of anti-GPI IgGs, possibly resulting in insufficient anti-GPI neutralizing activity. In contrast, there was no significant association of anti-MSP1p19 antibodies in these three categories of individuals. This may be related to differences in the kinetics of the production of antibodies against GPI and MSP1p19. Glycolipids are generally poor antigens compared to proteins and therefore, the anti-GPI antibody response may require repeated exposure, as compared to rapid boosting of the highly immunogenic MSP1p19 antigen. Even though the anti-GPI antibody responses are considerably lower than those for MSP1p19, they nevertheless seem to influence significantly the development of cerebral malaria.

The presence of comparable levels of anti-MSP1p19 antibodies in individuals from both study groups, regardless of disease status, suggests that protective responses against parasite surface protein antigens, which likely control infection by interfering with parasite invasion

[17], may not be sufficient for effective control of malaria. Thus, the results of our study argue in support of the widely prevailing notion that protective immunity against malaria pathology consists of two major components: “antiparasite” and “antitoxin” immunity [25]. While antibodies against parasite antigens such as MSP1p19 can effectively control parasite infection [17, 19], and thereby lower the risk of developing severe malaria, anti-GPI antibodies, have been suggested to provide protection against the development of severe disease by neutralizing the activity of GPIs [2]. Although in some cases, depending on host genetic variation, the anti-parasite approach may provide sufficient protection, the combined approach is expected to be much more effective. Thus, the anti-GPI antibody response can be an important and valid target for the development of anti-disease therapies and/or vaccines. However, since anti-GPI antibodies are short lived, the challenge is to obtain long lasting antibody responses to realize the full potential of GPI-based vaccines [10, 12].

An unexpected finding of this study is that, despite elevated mean IgG responses to MSP1p19 in all malaria infected groups, there were significantly lower IgG levels in the subgroup of individuals with fatal outcomes to cerebral malaria, compared to those who recovered. These results are consistent with recent findings of a prospective study in Ndiop suggesting a requirement for a critical level of anti-MSP1p19 antibodies (OD ratio = 7), for a significant association with delayed reinfection following drug cure [19]. In this study, anti-MSP1p19 IgG responses in fatal cases were unusually low compared to results of several studies of *P. falciparum* infected individuals (urban and hospital consultants) using the baculovirus expressed MSP1p19 antigen (unpublished data). However, specific IgG responses to MSP1p19 do not qualify as prognostic measures for predicting severe disease and/or fatal outcomes in infected individuals, because multiple targets on the merozoite surface are likely to contribute to the prevention of hyper-parasitemia. In addition, since anti-disease immunity is required to achieve complete protection against severe malaria [26], our results show that

measures of anti-GPI IgG could not discriminate between recovery and fatal outcomes to cerebral malaria.

In summary, our results argue for a substantial protection-associated role of anti-GPI IgGs against the manifestations of cerebral malaria. However, a direct role for anti-GPI antibodies in neutralizing parasite toxins involved in malaria pathogenesis remains to be demonstrated. Our results call for further studies on the mechanisms by which GPIs are able to stimulate host adaptive immune responses to produce anti-GPI antibodies to exploit the potential of this approach for malaria prophylaxis or therapy.

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7. Legends to Table and Figures

Table 1. Levels and prevalence of IgG responses against MSP1p19 and GPI in the different study groups

Figure 1: Antibodies against GPIs and MSP1p19 in study group 1. Histograms represent mean levels (\pm SE) of IgG responses of 35 immune controls from Ndiop (dark grey), 35 adults with “mild” malaria (light grey), and 35 patients with confirmed cerebral malaria (black). Shown are the levels of IgG antibodies against GPI (a) and MSP1p19 (b). The asterisks (*) indicates $P < 0.05$.

Figure 2: Antibodies against MSP1p19 and GPI in study group 2. Histograms represent mean IgG levels (\pm SE) against MSP1p19 (a, c) and GPI (b, d) for 47 non-infected individuals (a and b), 63 immune controls from Ndiop (a and b), 30 individuals with “mild” malaria (a and b), and 70 patients with cerebral malaria (a, b and c). Shown are the levels of IgG antibodies to MSP1p19 (a, c) and GPI (b). The asterisks (*) indicates $P < 0.05$.

Figure 3: Relationship between age and antibody responses against MSP1p19 and GPI in study group 2. Shown are the age- related distribution of Ab responses against MSP1p19 (a, b) and GPI (c,d) of Ndiop individuals (a, c), and of patients with cerebral malaria (b, d). A significant relationship between age and IgG responses against MSP1p19 and GPI was found for Ndiop villagers ($P < 0.001$ by Spearman rank test) but not for hospitalized patients.

Table 1. Levels and prevalence of IgG responses against MSP1p19 and GPI in the different study groups

Category of individuals	group ^a	n	IgG responses against			
			MSP1p19		GPI toxin	
			mODrt ± SD ^b	Prev. ^c	mODrt ± SD	Prev.
Immune individuals (Ndiop)	1	39	9.1 ± 4.0	96%	3.2 ± 3.0	43%
Patients with mild malaria	1	35	7.7 ± 2.1	100%	2.6 ± 3.3	31%
Patient with cerebral malaria	1	35	7.2 ± 2.5	94%	1.7 ± 0.8	17%
Immune individuals (Ndiop)	2	63	8.0 ± 4.1	87%	3.3 ± 3.4	43%
Patients with mild malaria	2	30	6.6 ± 4.4	87%	2.8 ± 3.3	33%
Patient with cerebral malaria	2	70	6.7 ± 4.4	71%	1.5 ± 0.9	14%
Urban negative controls	2	47	1.7 ± 1.6	4%	1.1 ± 0.2	0%

^a Study groups of individuals: 1 & 2 = first and second group independently investigated

^b Mean OD ratio ± standard deviation

^c Prevalence of positive responders *ie* individuals with an OD ratio > 2

Figure 1

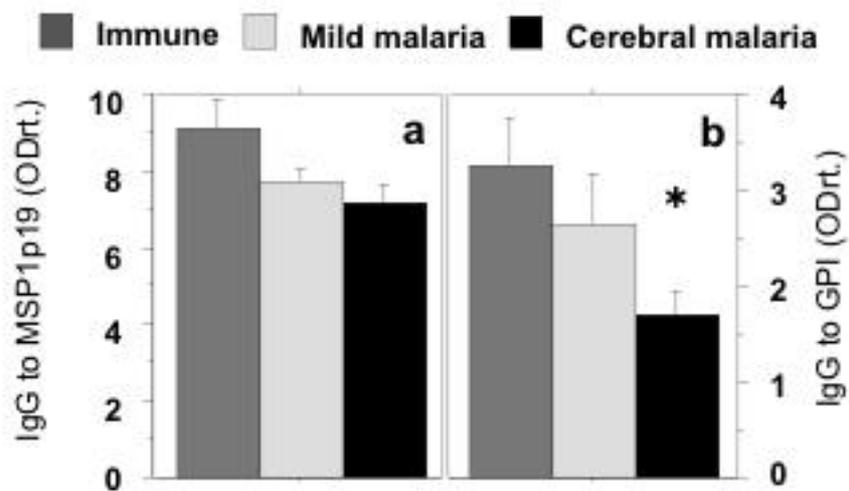


Figure 2

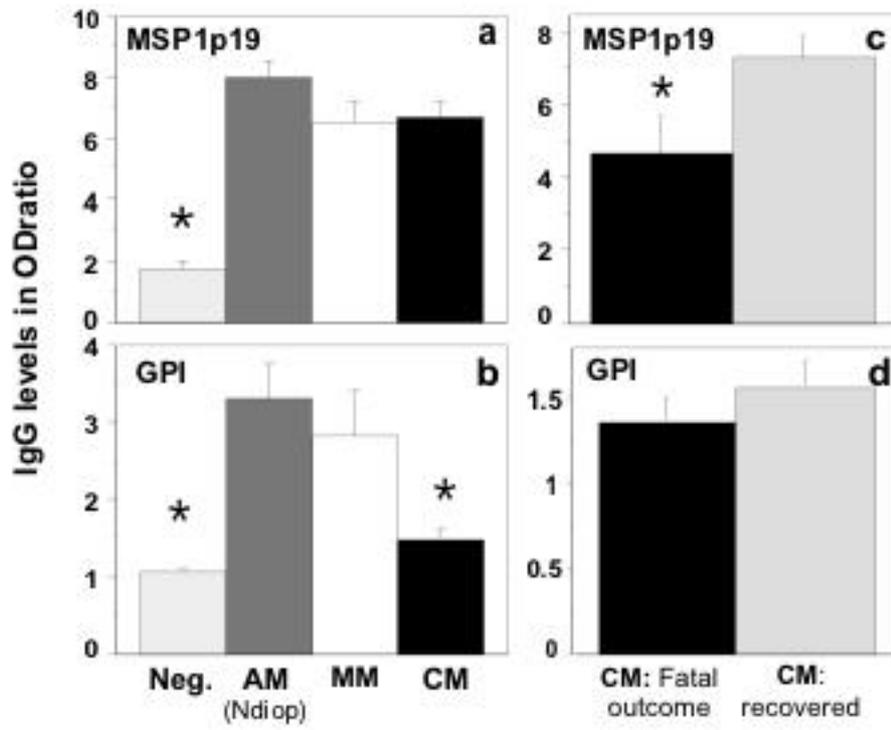


Figure 3

