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SERUM ANTIBODY LEVELS TO GLYCOSYLPHOSPHATIDYLINOSITOLS IN SPECIMENS DERIVED FROM MATCHED MALIAN CHILDREN WITH SEVERE OR UNCOMPLICATED *PLASMODIUM FALCIPARUM* MALARIA AND HEALTHY CONTROLS

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Abstract

Neutralizing antibodies to glycosylphosphatidylinositols (GPIs), which are *Plasmodium falciparum* surface protein anchor molecules implicated in malaria pathogenesis, are thought to protect against symptomatic malaria. Index cases of severe malaria in Malian children 3 months to 14 years of age were matched by age and residence to uncomplicated malaria and healthy controls. Serum antibodies to GPI (IgM and IgG) were measured at the time of severe malaria and after the malaria transmission season. The mean optical density values for IgM and IgG antibodies were higher in children with severe or uncomplicated malaria compared with healthy controls. Similarly, higher percentages of children with IgM and IgG antibodies to GPI were observed in the severe malaria group compared with matched healthy controls. IgG antibody levels to GPI were highest among children with cerebral malaria and children who died. The IgG antibody levels to GPI peaked during periods of malaria transmission and decreased after malaria transmission ended. A direct correlation between age and parasitemia and IgG antibodies to GPI was observed. In summary, higher levels of IgM and IgG antibodies to GPI in young children were associated with disease severity and were short-lived.

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INTRODUCTION

Pro-inflammatory cytokine responses are partially responsible for many of the clinical manifestations of acute *Plasmodium falciparum* malaria infection.^{1–3} The stimulus leading to this cytokine cascade is incompletely understood but may derive from soluble parasite moieties or toxins released at the point of schizont rupture and merozoite release. Membrane anchors, known as glycosylphosphatidylinositols (GPIs), link malaria surface proteins (e.g., circumsporozoite protein, merozoite surface proteins 1, 2, and 4) to cell membranes and may be important mediators of tumor necrosis factor- α (TNF- α) production by macrophages and adhesion expression in vascular endothelium.^{4–6} These glycolipids are ubiquitous in many parasitic species (e.g. *Leishmania*, *Trypanosoma*, and *Plasmodium*)⁶ and are produced in excess of what is required for membrane anchoring.⁷ The innate immune response elicited by GPIs occurs through the recognition of specific molecular patterns on the plasmodium by toll-like receptors (specifically TLR2 and to a lesser extent TLR4) which trigger specific cell signaling pathways resulting in production of TNF- α , interleukin-6 (IL-6), IL-12, and nitric oxide.⁸ Regulation of this activity can occur through GPI degradation by macrophage phospholipases.⁹ Antibodies to GPI may contribute to the acquisition of immunity to malaria and ameliorate malaria pathogenesis.¹⁰ Furthermore, vaccines targeting these toxins may be useful in abrogating disease associated with malaria infection, as has been demonstrated in mice.¹¹

Age-specific malaria immunity reduces the systemic effects of malarial disease but a relationship between antibodies to GPI and protection from disease has not been firmly established. Although IgG antibody to GPI was correlated with protection from malarial-induced anemia and fever in Kenyan children, protection from symptomatic diseases was not established in Gambian children or Kenyan adults.^{10,12} Low levels of IgG antibody to GPI were demonstrated in a mixed population of Senegalese adults and children with cerebral malaria compared with uncomplicated malaria, suggesting that lack of antibody correlated with disease susceptibility.¹³ Conversely, high levels of antibody were detected in Gambian children with malaria, which may reflect a rapid boosting effect of erythrocytic infection upon production of antibodies to GPI.¹² Rapidly produced antibodies to GPI have been shown to be short-lived and of the IgG₃ subclass.¹⁴ To clarify discrepancies in production of antibodies to GPI, we examined IgM and IgG antibody production in Malian children in a case-control study designed to detect correlates of protection from severe malaria.^{3,15,16}

METHODS AND MATERIALS

Study site and enrollment

The study was conducted in Bandiagara, Mali, a town with approximately 13,600 inhabitants in the northeastern Malian Sahel. This area has intense seasonal transmission of *P. falciparum* malaria, with cases of severe malaria occurring from July to January. We defined the early transmission season as July–September; the middle transmission season as October–November, and the end transmission season as December–January. Transmission usually starts in July and peaks in September or October, with the number of infected bites per person per month ranging from 20 to 60 in Bandiagara town.¹⁵ Transmission then decreases to low levels by December and is virtually undetectable during the January–June dry season.¹⁷ The dominant ethnic group is Dogon (80%) with the remainder of the inhabitants being Peuhl (10%), Bambara (3%), or others (7%).

Serum was obtained from Malian children (age range = 3 months to 14 years) on enrollment into a case-control study evaluating risk and protective factors for severe malaria. Index cases of severe malaria from Bandiagara and surrounding areas were admitted over the course of three malaria transmission seasons (1999–2001). Cases were classified as severe malaria based

on modified criteria of the World Health Organization (WHO).¹⁸ Each index case was age-, residence-and ethnicity-matched to a case of uncomplicated malaria and a healthy control. Age categories were defined as 3–5 months, 6–11 months, 1 year, 2 years, 3–4 years, 5–6 years, 7–8 years, 9–10 years, 11–12 years, and 13–14 years. Residence was defined as one of eight distinct sectors of Bandiagara town or, in the case of children from outer villages, the specific village of origin. Uncomplicated malaria was defined as *P. falciparum* parasitemia and an axillary temperature $\geq 37.5^{\circ}\text{C}$ detected by active surveillance, or parasitemia and symptoms leading to treatment-seeking behavior in the absence of other clear cause of fever on passive surveillance. Matched uncomplicated malaria controls were enrolled from the population of children coming to a daily clinic. Healthy controls were enrolled after traveling to the home of the child with severe malaria and following a standardized routine of exiting the front entrance of a compound and making consecutive left turns until another compound with an eligible control was identified. Children were enrolled as healthy controls if they were asymptomatic for acute illness, had no evidence or history of chronic illness and, if they were found to be aparasitemic upon examination. Controls were enrolled within 3–5 days of the index case enrollment. A post transmission season follow-up appointment for children enrolled during the previous wet season was conducted in April of each year with sera collected at this second time point.

Study protocols were reviewed and approved by the University of Mali and University of Maryland Institutional Review Boards. Community permission for the study was obtained as described.¹⁹ Individual informed consent was obtained from the legal guardian of each child prior to enrollment. Care for severe and uncomplicated malaria was offered regardless of study participation.

Specimen collection

Patient whole blood (1 mL) was collected into sterile Eppendorf (Hamburg, Germany) tubes on admission and before beginning anti-malarial therapy. Blood was allowed to coagulate for 4–6 hours prior to processing by centrifugation and stored at -20°C until specimen processing.

Antibody assays

IgG and IgM antibodies to GPI were measured by an optimized enzyme-linked immunosorbent assay (ELISA). The GPI used in this study were isolated and purified by high-performance liquid chromatography (HPLC) as previously described.¹⁰ Briefly, *P. falciparum* cultures at parasitemias of 35–40% were harvested and the parasites were released with 0.05% saponin in Trager's buffer. The erythrocyte membrane fragments were removed from the parasites by density centrifugation on cushions of 5% bovine serum albumin in Trager's buffer, and the purified parasites were lyophilized. To monitor GPIs during the isolation and purification steps, the parasite preparation was mixed with an aliquot of parasites metabolically labeled with ^3H -glucosamine. The parasites were extracted with chloroform/methanol (2:1 [v/v]) followed by chloroform/methanol/water (10:10:3 [v/v/v]). The latter organic extract containing the GPIs was dried and the residue was partitioned between water and water-saturated 1-butanol. The 1-butanol layer was dried and the GPIs were purified by reversed-phase HPLC using a C4 Supelcosil column (4.6 $\times$ 250 mm, 5- μm particle size; Supelco, Bellefonte, PA). The purity of the several batches of GPIs prepared by this procedure was $> 95\%$ as judged by mass spectrometry. The purified GPIs were quantitated by determining their mannose and glucosamine contents by high-pH anion-exchange HPLC of the aliquots of samples hydrolyzed with 2.5 trifluoroacetic acid at 100°C for 4 hours or 3 M HCl at 100°C for 3 hours.²⁰

GPI was diluted in methanol, coated onto 96-well microtiter plates at a concentration of 0.5 ng/well for both IgG and IgM assays, and incubated overnight at 25°C . Plates were washed with phosphate-buffered saline and 0.05% Tween 20 (used for all subsequent washes) and

blocked with 150 μ L of Tris-buffered saline/0.5% Tween 20 containing 5% casein at 25°C for 1 hour. (Note that in the absence of the addition of detergent to the washing buffer, high background created by non-specific antibody binding resulted in erroneous antibody levels. Therefore, 0.05% detergent was included in the washing buffer. ^3H -labeled GPI coating studies have detected no GPI loss occurring after incubation with diluted sera.) Samples and controls were added at a 1:100 dilution in duplicates for IgG to GPI-coated and uncoated wells and incubated for 2 hours at 25°C. Plates were washed and their contents incubated with 50 μ L of horseradish peroxidase-conjugated goat anti-human IgG (heavy and light chains; Kirkegaard and Perry Laboratories, Gaithersburg, MD) at a 1:2,000 dilution for 1 hour at 25°C. Optical densities (ODs) were read at 405 nm after 60 min of incubation with 50 μ L of 2,2'-azino-di-3-ethyl-benzthiazoline-6-sulfonate) substrate (Kirkegaard and Perry Laboratories).

Background ODs from uncoated wells were subtracted from those of GPI-coated wells to adjust for nonspecific binding. Sera from malaria-naïve volunteers from the United States were used as negative controls and sera from Malian adults were used as positive controls. Positive responders were defined as individuals with an OD > 3 SE above the mean of unexposed U.S. controls. All sera differing in value by $\geq 20\%$ in one or more wells were retested. A second ELISA was conducted on the available dry season sera of subjects who were positive during the wet season to assess the persistence of IgG level over time.

Data analysis

Pooled analyses of differences in GPI levels between clinical groups were performed using unadjusted a two-sided Student's *t*-test for continuous variables with equal variance (SPSS version 10.0; SPSS, Inc., Chicago, IL) and Mann-Whitney rank sum analysis for populations not normally distributed (SigmaStat version 3.0; SPSS, Inc.). Chi-square analysis was used for proportional analysis (EpiInfo 2000; Centers for Disease Control and Prevention, Atlanta, GA). For the purposes of analyzing differences in GPI levels, the level for statistically significant differences (two-sided) was set at $P < 0.05$.

RESULTS

Antibody production by group

A total of 208 index cases of severe malaria (mean age = 39.5 months, range = 3–162 months) were enrolled and each was matched to a case of uncomplicated malaria and a healthy control. Serum was available for analysis in 197 severe malaria cases, 206 uncomplicated malaria cases, and 203 healthy controls. The mean level of IgM antibody to GPI was higher in children with severe or uncomplicated malaria than in healthy controls (0.182 versus 0.104; $P < 0.001$ and 0.142 versus 0.104; $P < 0.001$, respectively). Similarly, the mean level of IgG antibody to GPI was higher in children with severe malaria than in healthy controls (0.199 versus 0.134; $P = 0.003$) and in children with uncomplicated malaria than in healthy controls (0.218 versus 0.134; $P = 0.018$). The proportion of IgG-positive sera between matched pairs was higher in the severe malaria group (57 of 197, 28.9%) than in healthy controls (40 of 203, 19.7%) (odds ratio [OR] = 1.66, 95% confidence interval [CI] = 1.02–2.71, $P = 0.03$) (Table 1). No difference was observed between the severe and uncomplicated malaria groups or between uncomplicated malaria patients and healthy controls. The proportion of IgM-positive sera was higher in children with severe malaria (108 of 197, 54.8%) and those with uncomplicated malaria (92 of 206, 44.7%) than in healthy controls (63 of 203, 30.5%) (OR = 2.70, 95% CI = 1.76–4.15, $P < 0.001$ and OR = 1.79, 95% CI = 1.17–2.74, $P = 0.005$, respectively). No differences were observed between severe and uncomplicated malaria cases.

Age-related antibody production

Linear regression models demonstrated a modest correlation between mean OD of IgG antibody to GPI and age in months (all participants: $R = 0.208$, $P < 0.001$, severe malaria: $R = 0.183$, $P = 0.01$, uncomplicated malaria: $R = 0.252$, $P < 0.001$, and healthy control: $R = 0.172$, $P = 0.014$). In addition, a general trend of increasing mean ODs of IgM and IgG antibodies to GPI and antibody seropositivity was noted after age stratification of results (Figures 1 and 2). Children from all groups were stratified into those with IgG-positive sera who were ≥ 5 years of age and those < 5 years of age, with older children having a higher seropositivity than younger children (43 of 120 [35.8%] versus 108 of 484 [22.3%]; $P = 0.003$). Similarly, significantly more children ≥ 5 years of age with severe malaria had IgG-positive sera compared with children < 5 years of age (17 of 40 [42%] versus 40 of 156 [25%]; $P = 0.036$). No difference in the number with IgG-positive sera was noted in children with uncomplicated malaria or healthy children when stratified by age. IgM antibody production appeared diminished in children less than 12 months of age in all groups but no additional differences were noted in mean OD of IgM antibody to GPI after age stratification. No difference in the distribution of IgM-positive sera was noted in the severe malaria or uncomplicated malaria enrollment groups after age stratification, but the proportion of positive sera was significantly higher among healthy controls ≥ 5 years of age than in those < 5 years of age (19 of 41 [46.3%] versus 43 of 161 [26.7%]; $P = 0.015$).

Intra-seasonal and seasonal variation of antibody response

The distribution of IgM and IgG antibodies to GPI was analyzed after stratification into those collected early in the transmission season, in the middle of the transmission season, or at the end of the transmission season. Detectable levels of serum IgM antibody to GPI were significantly higher in healthy children enrolled early and in the middle of the transmission season compared with those enrolled at the end of the season ($P = 0.03$ and $P = 0.04$, respectively) (Figure 3a). No significant differences in IgM antibody to GPI were detected for either the severe or uncomplicated malaria groups. Detectable levels of IgG antibody to GPI were greatest in midseason, which corresponded to the peak of malaria transmission compared with the early transmission season for all cohorts (severe malaria: 0.255 versus 0.145; $P = 0.02$, uncomplicated malaria: 0.297 versus 0.098; $P < 0.001$, healthy controls: 0.174 versus 0.104; $P = 0.04$). Likewise, the mean OD of midseason was elevated over that measured at the end of the season for all cohorts (severe malaria: 0.255 versus 0.169; $P = 0.046$, uncomplicated malaria: 0.297 versus 0.180; $P = 0.02$, healthy controls: 0.174 versus 0.106; $P = 0.067$) (Figure 3b).

An antibody measurement was made in the dry season for all children who were positive for IgG antibody to GPI in the transmission season. Short-lived antibody response was noted with a high proportion of children reverting to IgG negative. The mean OD IgG value also decreased sharply post-transmission season compared with during the transmission season in all groups ($P < 0.001$) (Table 2). Few children had residual asymptomatic malaria infection during the dry season.

Antibody level and disease severity

To determine whether the level of antibody to GPI correlated with disease presentation, severe malaria cases were stratified by common WHO criteria for enrollment (cerebral malaria [Blantyre coma scale ≤ 2]), seizure, or obtundation) ($n = 89$), severe anemia (hemoglobin level ≤ 5 g/dL) ($n = 33$), or hyperparasitemia ($\geq 500,000$ asexual parasites/mm³) ($n = 75$). No difference was noted in the percentage of IgM- or IgG-positive sera or mean antibody OD values when stratified by specific severe malaria disease criteria and compared with other severe malaria cases. However, elevated levels of IgM were noted in children with cerebral malaria compared with cases of uncomplicated malaria (0.219 versus 0.142; $P = 0.008$).

Moreover, elevated levels of both IgM and IgG were noted in children with cerebral malaria compared with children without malaria (IgM: 0.219 versus 0.104; $P < 0.001$, IgG: 0.232 versus 0.134; $P = 0.001$). Children who died of severe disease had elevated IgM and IgG levels compared with all other groups although with the small number of deaths this was only significant compared with healthy controls. No elevation of IgM or IgG levels was noted in other sub-classifications of severe malaria compared with uncomplicated cases of malaria or healthy controls. Multiple linear regression models showed that in addition to age (in months), absolute parasitemia in the severe malaria cases correlated with the mean OD value of IgG ($P = 0.007$). When all groups were combined, a trend towards correlation of parasitemia and the mean OD of IgG ($P = 0.05$) was noted.

DISCUSSION

In a matched case-control study of severe *P. falciparum* malaria in an area of intense seasonal malaria transmission, the production of antibodies to GPI was modestly lower in healthy children compared with children with uncomplicated or severe malaria, and higher in children with cerebral malaria compared with those with other manifestations of severe malaria. Antibody production appeared rapidly with disease onset, peaked during the midseason when malaria transmission was highest, and decreased rapidly with most children becoming seronegative during the subsequent dry season. Furthermore, IgG antibody production increased with age and correlated with parasitemia in the severe malaria cohort.

Longitudinal studies have demonstrated that children rapidly acquire modest level of antibody to GPI after a single malaria infection and incrementally acquire additional antibody with successive infections. In contrast, adults appear to develop high levels of antibody at the time of first infection and are able to sustain antibody levels longitudinally.²¹ Levels of antibodies to GPI were elevated in both severe and uncomplicated malaria cases compared with healthy controls, suggesting that antibody production increases with disease severity. High-titer antibody levels have been found in West African children and attributed to a rapid boosting effect of antibody production by malaria infection.¹² This antibody response appears short-lived, accounting for the low level of antibody detected during the post-transmission follow-up. The high antibody levels noted at the peak of the transmission season may provide further evidence of the close association of infection and antibody development in this young population. Incremental exposure to infection over time may result in age-associated increases in antibody levels. Case-control studies are well suited for studying risk factors for relatively uncommon diseases such as severe malaria, but a prospective, longitudinal cohort study would be ideal to confirm the hypothesis that antibodies to GPI confer protection against the acquisition of disease.

We stratified antibody production by severe disease syndromes, postulating that different manifestations of severe malaria might be associated with defined levels of antibodies to GPI. Contrary to data demonstrating a protective effect of IgG antibodies to GPI with elevated levels noted in adult patients with uncomplicated malaria compared with hospitalized patients with cerebral malaria,¹³ we found elevated levels of both IgM and IgG antibodies to GPI in children with cerebral malaria compared with healthy controls. Other severe malaria disease sub-classifications did not have different antibodies to GPI compared with cases of uncomplicated malaria or healthy controls. Although it is possible that healthy controls had lower antibody levels because of reduced malaria exposure, the effects of heterogeneous malaria transmission were minimized by matching cases and controls by age and residence. Moreover, nearly all young children in Bandiagara have one or more clinical malaria episodes every season and it is highly unlikely that any are not exposed to malaria infection.¹⁷ It is conceivable that this healthy population may have been skewed towards children with other, as yet undetermined factors for malaria resistance, because only aparasitemic healthy controls were enrolled.

Some studies have shown an inverse correlation of antibodies to GPI and parasitemia.²² As malaria immunity is acquired, tolerance to parasitemia increases, leading to asymptomatic malaria infections. Absolute parasitemias decrease with age.²² The WHO recommends the use of hyperparasitemia as a criterion for severe malaria, but the clinical course in children with this manifestation appears to be less acute. At our study site, children whose sole defining criterion for severe malaria is hyperparasitemia appear to fall somewhere between uncomplicated malaria and severe malaria in the spectrum of illness and are generally older than the mean study age of 39.5 months. Given the high number of children enrolled as hyperparasitemic (38%), our findings that levels of antibodies to GPI are directly correlated with parasitemia are likely related to an age-associated correlation in acquisition of antibodies to GPI and not with disease severity. The young age of our study population precludes any determination of age-associated decreases in parasitemia.

In summary, the case-control design of this study allowed us to examine the relationship between levels serum antibodies to GPI in matched cohorts of children distributed among different stages of malarial disease. Our data confirm previous findings suggesting that production of antibodies to GPI is rapid, short-lived, and cumulative in young children in malaria-endemic regions. However, they do not support a role of antibodies to GPI in protection against disease. Prospective, longitudinal cohort studies would be required to determine the role of antibodies to GPI in naturally acquired immunity to malaria.

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Figure 1. Mean optical density (O.D.) values of **a**, IgM and **b**, IgG antibodies to glycosylphosphatidylinositol (GPI) in all enrolled Malian children stratified by age in months and by age- and residence-matched disease cohort (severe malaria, uncomplicated [mild] malaria, and healthy controls). Error bars show the standard error (SE). The threshold for seropositivity (> 3 SE of the mean of unexposed volunteers) is indicated by the dotted horizontal line.

Figure 2. Percentage of Malian children positive for **a**, IgM **b**, IgG antibodies to *Plasmodium falciparum* glycosylphosphatidylinositol (GPI). The results are stratified by age in months and by age-and residence-matched disease cohort (severe malaria, uncomplicated [mild] malaria, and healthy controls). The number of children in each age group is indicated.

Figure 3.

Mean optical density (O.D.) values of **a**, IgM and **b**, IgG antibodies to glycosylphosphatidylinositol (GPI) in Malian children with severe or uncomplicated [mild] malaria or healthy controls based on the period of enrollment (Early = July–September, Mid = October–November, and End = December–January) within the transmission season. The asterisk in **a** indicates a significant elevation in early transmission compared with both the middle and the end of the transmission season ($P < 0.05$). The asterisk in **b** indicates a significant elevation in midseason compared with the early season in the severe and uncomplicated malaria cohorts and healthy controls ($P < 0.05$). A significant difference between the mean midseason OD and the mean end season OD was observed for the severe

and uncomplicated malaria cohort groups but not the healthy controls. Error bars show the standard error (SE). The threshold for seropositivity (>3 SE of the mean of unexposed volunteers) is indicated by the dotted horizontal line.

Table 1

Patient characteristics of Malian children with matched severe or uncomplicated malaria or healthy controls at enrollment*

Characteristics	Severe malaria		Uncomplicated malaria	Healthy controls
	Survived	Died		
No. (%)	182 (92.4)	15 (7.6)	206	203
Female (%)	85 (46.7)	8 (53.3)	102 (49.5)	90 (44.3)
Age in months (range)	40.3 (3–162)	29.9 (8.3–99.3) [†]	39.8 (3.0–168)	38.1 (3.0–124)
IgM-positive sera (%)	98 (53.8)	10 (66.7)	92 (44.7)	62 (30.5) ^{‡§}
Mean anti-GPI IgM (OD)	0.179	0.227	0.142	0.104 ^{‡§}
IgG-positive sera (%)	52 (28.6)	5 (33.3)	54 (26.2)	40 (19.7) [‡]
Mean anti-GPI IgG (OD)	0.192	0.290	0.218	0.134 ^{‡§}
Hemoglobin (g/dL) [†] (range)	8.49 (2.6–14.2)	6.18 (2.0–11.8) [†]	9.3 (5.3–14.2) [¶]	10.5 (6.2–14.5) ^{‡§}
Geometric mean parasite density/ μ L	195,053	40,073 [†]	10,497 [¶]	0 ^{‡§}

* GPI = glycosylphosphatidylinositol; OD = optical density.

[†] Denotes significance at the level of $P < 0.05$ between children who survived and those who died of severe disease. All analysis performed with paired Student's t -test.

[‡] Paired t -test or Mann-Whitney rank sum significance ($P < 0.05$) between children with severe malaria and healthy controls.

[§] Paired t -test or Mann-Whitney rank sum significance ($P < 0.05$) between children with uncomplicated malaria and healthy controls.

[¶] Paired t -test or Mann-Whitney rank sum significance ($P < 0.05$) between children with severe and uncomplicated malaria.

Table 2

Comparisons of anti-GPI IgG-positive sera and OD mean of antibody of individuals with both transmission (wet) and post-transmission (dry) season samples^{*}

Study groups	Positive anti-GPI IgG antibody No. (OD mean)		<i>P</i> [†]
	Wet season	Dry season	
Severe malaria	29 (0.580)	2 (0.233)	< 0.001
Uncomplicated malaria	38 (0.706)	1 (0.248)	< 0.001
Healthy controls	22 (0.562)	3 (0.195)	< 0.001

^{*} GPI = glycosylphosphatidylinositol; OD = optical density.

[†] By Wilcoxon signed rank test ($P < 0.05$) for wet season vs. dry season.