Antibodies to *Plasmodium falciparum* Glycosylphosphatidylinositols: Inverse Association with Tolerance of Parasitemia in Papua New Guinean Children and Adults

Craig S. Boutlis, D. Channe Gowda, Ramachandra S. Naik, Graeme P. Maguire, Charles S. Mgone, Moses J. Bockarie, Moses Lagog, Erwin Ibam, Kerry Lorry and Nicholas M. Anstey


Updated information and services can be found at: [http://iai.asm.org/content/70/9/5052](http://iai.asm.org/content/70/9/5052)

**REFERENCES**

These include:

This article cites 42 articles, 22 of which can be accessed free at: [http://iai.asm.org/content/70/9/5052#ref-list-1](http://iai.asm.org/content/70/9/5052#ref-list-1)

**CONTENT ALERTS**

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), [more»](http://iai.asm.org/content/70/9/5052#ref-list-1)
Antibodies to *Plasmodium falciparum* Glycosylphosphatidylinositol: Inverse Association with Tolerance of Parasitemia in Papua New Guinean Children and Adults

Craig S. Boutlis,1,2 D. Channe Gowda,3† Ramachandra S. Naik,3‡ Graeme P. Maguire,1 Charles S. Mgone,4 Moses J. Bockarie,5 Moses Lagog,5 Erwin Ibam,5 Kerry Lorry,5 and Nicholas M. Anstey†*

Department of Tropical Medicine and International Health, Menzies School of Health Research, Casuarina,1 and Northern Territory University, Darwin,2 Australia; Department of Biochemistry and Molecular Biology, Georgetown University Medical Center, Washington, D.C.;4 and Papua New Guinea Institute of Medical Research, Goroka,4 and Madang,5 Papua New Guinea

Received 18 April 2002/Returned for modification 25 May 2002/Accepted 11 June 2002

Individuals living in regions of intense malaria transmission exhibit natural immunity that facilitates persistence of parasitemia at controlled densities for much of the time without symptoms. This aspect of immunity has been referred to as malarial “tolerance” and is thought to partly involve inhibition of the chain of events initiated by a parasite toxin(s) that may otherwise result in cytokine release and symptoms such as fever. Antibodies to the candidate *Plasmodium falciparum* glycosylphosphatidylinositol (GPI) toxin have been viewed as likely mediators of such tolerance. In this study, the relationship between antibodies to *P. falciparum* GPIs, age, and parasitemia was determined in asymptomatic children and adults living in Madang, Papua New Guinea. The prevalence and intensity of antibody responses increased with age and were lowest in children 1 to 4 years old with the highest-density parasitemias. In children of this age group who were tolerant of parasitemia during the study, only 8.3% had detectable immunoglobulin G (IgG) and none had IgM antibodies to GPI. This suggests that anti-GPI antibodies are unlikely to be the sole mediator of malarial tolerance, especially in children younger than 5 years. Following antimalarial treatment, clearance of parasitemia led to a fall in anti-GPI IgG response in children and adolescents within 6 weeks. As anti-GPI antibodies potentially play a role in protecting against disease progression, our results caution against the treatment of asymptomatic parasitemia and suggest that generation of a sustained antibody response in children poses a challenge to novel antitoxic vaccination strategies.

The natural history of malaria in regions of endemicity is characterized by long periods of asymptomatic parasitemia punctuated by episodic clinical attacks that decrease in frequency with age (24, 33). This pattern has been explained by the acquisition of exposure-related natural (or “clinical”) immunity that has been viewed for over 60 years as comprising two major components: “antiparasitic” (i.e., the ability to control parasite densities) and “antitoxic” immunity (i.e., suppression of disease symptoms despite infection) (39). The ability of individuals from regions of high endemicity to tolerate persistent parasitemia without fever is considered to be a manifestation of antitoxic immunity (17, 32). As the threshold of parasitemia associated with fever has been shown to be age dependent and higher in children than in adults from geographically diverse locations (26, 32, 40, 45), it has been proposed that this aspect of antitoxic immunity is most efficient in childhood and declines with age (17).

Accumulating evidence has identified *Plasmodium falciparum* glycosylphosphatidylinositol (GPIs) as putative toxins that initiate a number of cellular events that contribute to malaria pathogenesis. Induction of the fever-producing cytokines tumor necrosis factor alpha (TNF-α) and interleukin-1 by mononuclear cells has been demonstrated in vitro (29, 34), and transient pyrexia has been induced in vivo through administration of *P. falciparum* GPIs to mice (34). *P. falciparum* GPIs have also been demonstrated to up-regulate expression of endothelial cell surface receptors implicated in cytoadherence to parasitized red cells (36) and to induce hypoglycemia (34)—events implicated in the pathogenesis of severe malaria (15). GPIs therefore represent an attractive immunological target for strategies aimed at ameliorating disease due to *P. falciparum* (43).

Monoclonal antibodies to *P. falciparum*-derived GPIs have been demonstrated to neutralize the TNF-α-inducing activity of whole-parasite extracts in vitro (37), and a monoclonal antibody recognizing phosphatidylinositol has been shown to inhibit TNF-α induction by geographically diverse strains (3). Polyclonal antibody raised in T-cell-deficient mice (5) and sera from infected human patients with both *P. falciparum* and *Plasmodium vivax* infection (4) have been reported to have similar activity. On the basis of these studies, it has been hypothesized that antibodies to GPIs play a role in mediating tolerance of parasitemia and that their production would par-
allel the densities of parasitemia observed in tolerant individuals (32).

We hypothesized that individuals living in a region of intense malaria transmission produce anti-GPI antibodies that are induced by infection with _P. falciparum_. As threshold levels of asymptomatic parasitemia are reported to decline with age (32, 40), we reasoned that the prevalence and level of _P. falciparum_ anti-GPI antibodies would be higher in children than in adults. We tested these hypotheses by measuring immunoglubulin G (IgG) and IgM responses against GPIs in different age groups and investigated the association between antibody production and parasitemia both cross-sectionally and longitudinally.

**MATERIALS AND METHODS**

**Study site.** Subjects were residents of two neighboring coastal villages (Haven and Midiba) located approximately 20 km north of Madang township, Papua New Guinea (PNG). The region is characterized by infection with all four human malaria species, and there is little seasonal variation in parasitemia rates (12). Residents are estimated to receive on average close to one infective bite per day (10), with transmission highest during the wet season from October to May (11).

**Study population.** The study was conducted between February and May 2000 with ethical approval from the PNG Medical Research Advisory Committee and the Ethics Committee of the Menzies School of Health Research, Darwin, Australia. Following informed consent, nonpregnant adults and children who were ≧ 1 year of age were screened using a clinical questionnaire administered in the local language (Tok Pisin); measurement of axillary temperature; and examination of a finger prick blood smear for malaria parasites. Enrollment was confined to strictly defined asymptomatic subjects, with the selective aim of including microscopically parasitemic and asexual parasitemic subjects and representation across different age groups. Participants were excluded from enrollment if they were febrile (axillary temperature ≧ 37.5°C) at screening or on two subsequent occasions over the next 24 h; had taken antimalarials within 1 week; or had a clinical history (fever, chills, sweats, headache, or myalgia) of recent (≦ 1 week) malaria infection.

Peripheral blood smears were repeated at the time of venous blood collection 24 h after initial screening to account for periodic fluctuation of _P. falciparum_ density in particular (8, 18), and the combined readings were used to categorize the parasite species present. Subjects with _P. falciparum_ infection alone or in combination with other species received a single dose of 25 mg of sulfadoxine per kg of body weight and 1.25 mg of pyrimethamine (Fansidar; Roche, Dee Why, Sydney, Australia) and, subjects with _P. vivax_, _Plasmodium malariae_, and/or _Plasmodium ovale_ were given three daily doses of chloroquine phosphate (Pharmamed, Malta/10 mg of kg. Subjects were followed up 6 weeks after enrollment using the same procedures outlined above.

**Sampling collection and processing.** Thick and thin blood smears from all screened and enrolled subjects were treated with a 4% Giemsa stain and were examined by a trained microscopist with over 15 years of experience (M. Lagog). The placental proportion of parasites was determined by a second microscopist (K. Lorry; 12 years of experience), and discrepant slides were reviewed by both microscopists to arrive at a final result. Venous blood was collected into sterile heparinized tubes (Becton Dickinson, Lincoln Park, NJ) and stored at 4°C overnight onto half of a 96-well polystyrene microtiter plate (Maxisorb by Costar, Cambridge, Md.) or sheep anti-human IgM (μ chain; Chemicon, Australia) at 1:2,000 dilution for 1 h at 25°C. Plates were washed five times, and their background optical densities from uncoated wells were subtracted from those of GPI-coated wells to adjust for nonspecific binding.

The optical densities from 15 non-malaria-exposed Australian adult controls (mean age, 28 years [range, 20 to 44 years]; 33% male) were expressed as percentages relative to malaria-exposed adult positive controls for both IgG and IgM. As the results in Australian controls were normally distributed in both assays, values of 2 standard deviations above the means were chosen as representative cutoff between positive and negative and were arbitrarily assigned a value of 1. The optical densities from all subjects, after controlling for nonspecific binding, were similarly initially expressed as percentages relative to the positive controls and then as multiples of the cutoff in Australian controls (i.e., values that were > 1 indicated positive results). Positive and negative controls were run on each plate and showed acceptable variability between assays. Longitudinally paired samples from the same individual were assayed concurrently on the same plate.

**Data analysis.** Statistical analysis was performed using Stata version 6.0 (Stata Corporation, Tex.). Age was stratified in subgroups consistent with earlier studies of malaria immunepidemiology (20). Logistic regression was used to model the relationship between antibody positivity, age, and parasitemia. Other proportions were examined with the χ² test or Fisher’s exact test (16). The intensity of antibody response was correlated with age using Spearman’s rank test. Changes in antibody response were analyzed longitudinally using the paired Student’s t test or Wilcoxon test as appropriate for the distribution of data. Two-sided P values of < 0.05 were considered to indicate statistical significance.

**RESULTS**

**Baseline characteristics.** Single blood smears from 424 children who were ≧ 1 year old (160 from Haven and 264 from Midiba; 48.4% male) were screened by microscopy to enable selection of subjects for enrollment. The proportion of subjects positive for any malaria parasite at screening was highest in the 5- to 9-year age group (70.3%) and for _P. falciparum_ in the 1- to 4-year age group (50.8%). The prevalence of parasitemia in different age groups, splenomegaly (82.7% in subjects who were ≦ 14 years old, with a peak of 91.7% in children who were 5 to 9 years old), and stated bed net use (86.2% overall; 98.1% in children who were 1 to 4 years old) was broadly consistent with that found in previous data reported from this region (12, 17).

_P. falciparum_ parasitemia was present in 116 (54%) of the 216 screened subjects who were initially enrolled into the study. Venous blood collection was cancelled for 10 subjects because of heavy rain, and another 20 were subsequently excluded (axillary temperature of ≦ 37.5°C in eight subjects; recent malaria history in 12 subjects). Characteristics of the 186 subjects included in the study are given in Table 1. After cross-checking and examination of the second smear, one or
more additional parasite species were found in 21 of 77 (27%) initially aparasitemic subjects and in 25 of 109 (23%) subjects who were parasitemic on their screening smear. Three axillary temperature readings were recorded in 151 subjects; two in 29 subjects; and one only in six subjects. It was not possible to collect a second venous blood sample from all subjects due to time constraints: follow-up samples taken at a median of 6 weeks after enrollment were available from 115 (62%) of the 186 subjects; and one only in six subjects. It was not possible to collect a second venous blood sample from all subjects due to time constraints: follow-up samples taken at a median of 6 weeks after enrollment were available from 115 (62%) of the 186 subjects; nine of whom met the exclusion criteria at this time point and were excluded from longitudinal analysis. Age, gender, and baseline parasitemia either alone or in combination with other parasites were not analyzed statistically due to the lower numbers of child and adolescent subjects positive for IgM antibodies (Fig. 2B).

Relationship between anti-GPI antibody seropositivity and age. The likelihood of anti-GPI IgG seropositivity increased significantly with age: subjects from successive age groups (1 to 4, 5 to 9, 10 to 14, 15 to 19, and ≥20 years) were 3.5 times likelier than their immediate predecessors to be anti-GPI IgG positive (odds ratio [OR], 3.5; 95% confidence interval [CI], 2.4 to 5.2; P < 0.001 [Fig. 1A]). The magnitude of this association was unaltered after controlling for the nonsignificant effect of parasitemia with Plasmodium falciparum and/or other malaria parasites.

Baseline IgM antibody responses were tested in 128 (69%) of the 186 included subjects. IgM antibodies to GPI were absent in all subjects tested who were < 5 years of age, and although they increased across successive age groups, IgM seroprevalence was much less than that of IgG (Fig. 1B). After controlling for the significant effect of P. falciparum parasitemia (below), subjects who were ≥20 years were 10 times likelier than younger subjects to be IgM antibody positive (OR, 10; 95% CI, 3.3 to 30.4; P < 0.001).

The proportion of subjects with higher-intensity IgG antibody responses increased across successive age groups (Fig. 2A). The intensity of anti-GPI IgG response was positively correlated with age, grouped 1 to 4, 5 to 9, 10 to 14, 15 to 19, and ≥20 years (Spearman’s correlation coefficient, 0.55; P < 0.001). IgM antibody responses also increased with advancing age but were not analyzed statistically due to the lower numbers of child and adolescent subjects positive for IgM antibodies (Fig. 2B).

Cross-sectional relationship between anti-GPI antibody response and parasitemia. There was no association between the prevalence of P. falciparum parasitemia and anti-GPI IgG seropositivity at enrollment. Twelve of 19 (63.2%) 1- to 4-year-old subjects had P. falciparum parasitemia, but only one (8.3%) had IgG and none had IgM. In contrast, 47 of 71 subjects (66.2%) who were ≥20 years old had no P. falciparum parasitemia in their blood smear, yet 42 (89.4%) had anti-GPI

### TABLE 1. Baseline characteristics of 186 subjects included in the study

<table>
<thead>
<tr>
<th>Age group (yr)</th>
<th>n</th>
<th>Subject mean age (yr [95% CI])</th>
<th>% Male</th>
<th>No. of subjects with combined parasitemia on paired blood smears a</th>
<th>Results for P. falciparum b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–4</td>
<td>19</td>
<td>2.8 (2.4–3.3)</td>
<td>26.3</td>
<td>9 3 0 0 0 4 0 0 0 3</td>
<td>n  %  P. falciparum (%)</td>
</tr>
<tr>
<td>5–9</td>
<td>36</td>
<td>7.2 (6.7–7.7)</td>
<td>50.0</td>
<td>13 7 3 2 0 5 0 1 1</td>
<td>n  %  P. falciparum (%)</td>
</tr>
<tr>
<td>10–14</td>
<td>37</td>
<td>11.8 (11.3–12.3)</td>
<td>54.1</td>
<td>10 6 4 1 1 4 1 3 0</td>
<td>n  %  P. falciparum (%)</td>
</tr>
<tr>
<td>15–19</td>
<td>23</td>
<td>17.0 (16.2–17.7)</td>
<td>39.1</td>
<td>11 2 1 1 1 0 0 0 0</td>
<td>n  %  P. falciparum (%)</td>
</tr>
<tr>
<td>20+</td>
<td>71</td>
<td>31.6 (29.1–34.6)</td>
<td>46.5</td>
<td>20 3 0 1 0 1 0 9 2</td>
<td>n  %  P. falciparum (%)</td>
</tr>
</tbody>
</table>

a Number of subjects with each species (or combined species) of parasite on examination of two consecutive daily blood smears. P.f = P. falciparum; P.v = P. vivax; P.m = P. malariae; P.o = P. ovale; and Neg = negative for parasites.

b Number (n) and percentage (%) of subjects with P. falciparum parasitemia either as the sole infecting parasite or in combination with other parasites.

c P. f/µl is the geometric mean of the highest-density Plasmodium falciparum parasitemia measured from two consecutive daily blood smears.

FIG. 1. Percentage of subjects positive for IgG antibodies to GPs (means ± standard errors [S.E.]) (A) and IgM antibodies (B). Diamonds represent the percentage of subjects with P. falciparum (Pf) parasitemia either alone or in combination with other parasites for each age group; squares represent the geometric mean density of parasitemia (solid line).
IgGs. No association was observed between blood smear positivity for other malaria parasites and anti-GPI IgGs (data not shown), although these analyses may have been underpowered due to low numbers (Table 1).

In subjects aged ≥20 years, 56.5% (13 of 23) of subjects with \textit{P. falciparum} parasitemia (alone or in combination with other parasites) were IgM positive compared to 25% (9 of 36) without \textit{P. falciparum} (including aparasitemic subjects [OR, 3.9; 95% CI, 1.3 to 11.9; \(P = 0.015; \chi^2\) test]). Excluding subjects with mixed infections, anti-GPI IgMs were present in 55.6% (10 of 18) of subjects aged ≥20 years with \textit{P. falciparum} parasitemia, compared to 0 of 7 subjects with \textit{P. vivax} (\(P = 0.02; \) Fisher's exact test). The relationship between anti-GPI IgM positivity and parasitemia was not examined in subjects aged <20 years due to the small number of subjects positive for IgM antibodies (5 of 69; 7.2%).

\textbf{Longitudinal antibody response to GPI following clearance of parasitemia.} Paired antibody responses were examined in subjects whose baseline \textit{P. falciparum} parasitemia was cleared by treatment with standard antimalarials (verified on two consecutive daily blood smears after 2 weeks) and in whom no recrudescence or reinfection with \textit{P. falciparum} was noted at follow-up. All 18 seronegative subjects with \textit{P. falciparum} parasitemia at baseline remained seronegative at follow-up and were therefore not included in this analysis.

Eradication of \textit{P. falciparum} parasitemia was associated with a mean fall in IgG antibody response of 30% (95% CI, 17 to 43%) relative to baseline after a median of 6 weeks (interquartile range, 5 to 8 weeks; \(P < 0.001\)) in 31 subjects who were initially antibody positive. Antibody responses decreased in 17 of 19 subjects aged ≥20 years by a mean of 48% (95% CI, 36 to 60.1%; \(P < 0.001\)) but were unchanged in the 12 subjects aged ≥20 years (median increase, 1%; \(P = 0.39\)). There was no change in IgG responses in 26 control subjects without \textit{P. falciparum} parasitemia at either time point, either overall (mean decrease, 3.6%; \(P = 0.55\)) or in age-based subgroups.

\textbf{DISCUSSION}

This study demonstrates for the first time that, in a cohort of children and adults with intense malaria exposure, the presence of anti-GPI antibodies is directly associated with \textit{P. falciparum} parasitemia. Eradication of asymptomatic \textit{P. falciparum} infection in subjects who were <20 years of age was followed by a decrease in IgG antibody responses, whereas there was no change in older subjects. Blood smear positivity with \textit{P. falciparum} was associated with the presence of IgM antibodies to GPIs in subjects aged ≥20 years. Together these observations indicate that \textit{P. falciparum} can induce both IgM and IgG antibodies to its GPIs and that the IgG response is more persistent in adulthood.

There are a number of possible reasons for the lack of a cross-sectional association between blood smear positivity for \textit{P. falciparum} and anti-GPI IgGs. Children and adolescents aged 4 to 14 years from Madang Province have frequent sub-patent infections (i.e., PCR positive/microscopy negative) (9) that could induce antibody production, as do adults from a nearby region (25). Almost all subjects who were ≥15 years old were IgG positive, which is likely to reflect an increasing persistence of antibody response between infections, thus making an association with parasitemia more difficult to detect. The association between anti-GPI IgM and parasitemia in subjects aged ≥20 years may have been more evident due to an increased likelihood of IgM responses coinciding with infection, as IgM responses are generally of shorter duration than those of IgG (21).

Although the numbers were relatively small, we could find no evidence to support previous suggestions that \textit{P. vivax} induces antibodies that are cross-reactive with \textit{P. falciparum} GPIs (4, 6). In our study, IgM anti-GPI antibody responses were absent in subjects aged ≥20 years with \textit{P. vivax} infection but were present in a majority of subjects with \textit{P. falciparum} infection and in almost one-third of those who were aparasitemic. The positive responses seen in aparasitemic subjects...
may reflect recently eliminated and/or subpatent *P. falciparum* infection. In contrast, the lack of IgM response in those with *P. vivax* is consistent with the recent demonstration of species-transcending regulation of parasite density in this region, which results in significantly more sequential interspecies infections than in concurrent ones (7).

The prevalence and intensity of anti-GPI IgG and IgM responses to purified GPIs in the study population were positively related to age, consistent with a recent report from Kenya (29). This pattern was the inverse of mean parasitemia levels and also of the negative relationship previously demonstrated between age and parasite density in self-reporting febrile cases from Madang (17). In two other studies of populations resident in a nearby region in PNG (40) and an African region of holoendemicity (32), children under 5 years of age (although suffering more frequent clinical attacks) were shown to tolerate higher levels of parasitemia during asymptomatic infections than were older children and adults. These observations are consistent with earlier reports (26, 45) and support the general view that the ability to regulate parasite densities at lower levels during asymptomatic infections increases with age but that antitoxic immunity diminishes, as reflected by a decreasing fever threshold (17). If this is correct, then our finding that anti-GPI antibody responses were uncommon in tolerant children aged <5 years but were abundant in adults would suggest that these antibodies are unlikely to be the sole mediator of parasite tolerance and at most play a minor role in the youngest children.

Longitudinal studies may clarify what role anti-GPI antibodies play in natural immunity to malaria. As the pathophysiology of severe malaria is dependent on cytoadherence and local cytokine production (24), it is possible that these antibodies act to prevent disease progression by down-regulating the processes that lead to both of these events (34, 36). This potential role is consistent with recent epidemiological interpretations of the nature of clinical immunity to malaria (19, 41), and our data suggest that it would be likely to be more efficient in older children and adults than in children aged <5 years. Furthermore, our finding that anti-GPI antibodies increase with age concurrent with a decline in parasite density raises the possibility that these antibodies contribute to antiparasitic immunity. As the role of anti-GPI antibodies is presently unclear, our finding that anti-GPI IgG responses to *P. falciparum* are less persistent in children and adolescents provides further caution against the treatment of asymptomatic parasitemia in these age groups (13).

GPIs have been considered prime candidate molecules for vaccination strategies (43) that aim to diminish the manifestations of disease rather than protect against parasitemia (30). If anti-GPI antibodies can be shown to protect against disease progression, then generating a sustained antibody response in children <5 years of age will be a priority. Understanding the events involved in GPI antigen presentation and processing (28, 31, 35, 38) and whether they may be modified by adjuvants or immunomodulators (27, 44) may help improve vaccine immunogenicity in this age group. *P. falciparum* GPIs have been shown to induce production of nitric oxide by macrophages and endothelial cells in vitro (42), and systemic production of NO has been proposed to mediate tolerance of parasitemia (1, 2, 14) and to protect against uncomplicated and cerebral malaria (1) in young children. Active vaccination against GPI antigens may theoretically interfere with this mechanism of antitoxic immunity in addition to other potentially beneficial cytokine responses induced by GPIs (such as regulation of parasite density [23]).

In summary, our data show that individuals living in a region of high malaria endemicity produce in response to infection antibodies to *P. falciparum* GPIs that are more easily elicited, of higher intensity, and more persistent with increasing age. Our data suggest that other mechanism(s) of antitoxic immunity are likely to mediate tolerance of parasitemia in young children but do not exclude a role for anti-GPI antibodies in modifying the risk or outcome of clinical malaria in those individuals who produce the antibodies. Until the role of anti-GPI antibodies is clarified, our results caution against the clearance of asymptomatic parasitemia in children and adolescents in whom possible protective effects may be reduced by treatment. Longitudinal studies that correlate natural production of anti-GPI antibodies with disease risk and severity in different age groups may help to inform potential vaccination strategies targeting *P. falciparum* GPIs.

**ACKNOWLEDGMENTS**

This work was supported by the National Health and Medical Research Council, Australia (scholarship to C.S.B.); Tudor Foundation; Mark Nicholson and Alice Hill Malaria Research Fund; NIH RO1 grant nos. AI41764-04 and AI41139; and the Cooperative Research Centre for Aboriginal and Tropical Health, Australia.

We thank the people of Haven and Midiba villages for their participation and assistance; Joseph Slagi and Ferdinand Baighi for assisting with the fieldwork; and Andrew Raiko and his staff at the Madang IMR for facilitating the laboratory studies. We thank Michael Alpers, Bart Currie, Brice Weinberg, and Jodie Ridings for support.

**REFERENCES**

ANTIBODIES TO PLASMODIUM FALCIPARUM GPIs


Editor: W. A. Petri, Jr.