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

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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* glycosylphosphatidylinositols (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-

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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 immunoglobulin 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 aparasitemic 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, Australia)/kg, 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.

Specimen 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 field microscopist with over 15 years of experience (M. Lagog). Smears were defined as negative if no parasites were seen in 100 high-power (magnification, ×1,000) oil immersion fields. Positive slides with scanty parasitemias (≤5 parasites/200 leukocytes) and a random 10% of all slides from enrolled subjects were cross checked 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 from which a manual leukocyte count was performed for calculation of parasite densities (22). When a manual leukocyte count on the same day was unavailable (<25% of subjects), a subsequent manual count from the same person (taken 6 weeks later) was substituted, or an age-adjusted leukocyte count derived from the study population was used. Plasma was separated by centrifugation and stored at −70°C.

Antibody assays. Anti-GPI IgG and IgM antibodies were measured by enzyme-linked immunosorbent assay. The GPIs used in this study were purified by high-performance liquid chromatography (HPLC) of the *P. falciparum* GPI-enriched fraction obtained by solvent extraction procedures as described previously (29). The purity of the HPLC-purified GPIs was confirmed by carbohydrate compositional analysis and thin-layer chromatography of similarly purified fatty acid-labeled GPIs. The specificity of the seroreactivity of the HPLC-purified GPIs has previously been established (29). The GPIs were coated at 1 and 2 ng/well for IgG and IgM assays, respectively; these coating concentrations give saturated levels of seroreactivity. Tween 20 was included in the blocking buffer at a concentration that reduced nonspecific binding (which was evident in a number of subjects in preliminary experiments) without affecting the overall seroreactivity of samples or adherence of [^{3}H]glucosamine-labeled GPIs to the enzyme-linked immunosorbent assay plate (data not shown).

A stock solution of HPLC-purified GPIs was diluted with methanol and coated at 25°C overnight onto half of a 96-well polystyrene microtiter plate (Maxisorb by Nunc, N.Y.). Plates were washed once with 5% nonfat dairy milk in phosphate-buffered saline, pH 7.2 (PBS) containing 0.05% Tween 20 (used for all subsequent washes and dilutions) and then blocked with 250 μl of 5% nonfat dairy milk in PBS containing 0.5% Tween 20 at 25°C for 2 h. Fifty microliters of samples and controls (below) was added in duplicates at 1:100 dilution for IgG and at 1:25 for IgM to GPI-coated and uncoated wells on the same plate and were then incubated for 1 h at 25°C. Plates were washed five times, and their contents were then incubated with 50 μl of horseradish peroxidase-conjugated goat anti-human IgG (heavy and light chains; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) or sheep anti-human IgM (μ chain; Chemicon, Australia) at 1:2,000 dilution for 1 h at 25°C. Following five more washes (the last two in PBS, pH 7.2, containing 0.05% Tween 20), optical densities were read at a wavelength of 405 nm after 15 min of incubation with 50 μl of 2,2′-azino-di-(3-ethyl-benzthiazoline-6-sulfonate) substrate (Kirkegaard & Perry Laboratories). Finally, 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 representing a 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 immunoepidemiology (20). Logistic regression was used to model the relationship between antibody positivity, age, and parasitemia. Other proportions were examined with the χ^2 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

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

Age group (yr)	*n*	Subject mean age (yr [95% CI])	% Male	No. of subjects with combined parasitemia on paired blood smears[a]										Results for *P. falciparum*[b]		
				P.f	*P.f/P.v*	*P.f/P.m*	*P.f/P.v/P.m*	*P.f/P.v/P.o*	*P.v*	*P.v/P.m*	*P.m*	*P.o*	Neg	*n*	%	*P.f*/μl[c]
1–4	19	2.8 (2.4–3.3)	26.3	9	3	0	0	0	4	0	0	0	3	12	63.2	1,199
5–9	36	7.2 (6.7–7.7)	50.0	13	7	3	2	0	5	0	1	0	5	25	69.4	431
10–14	37	11.8 (11.3–12.3)	54.1	10	6	4	1	1	4	1	3	0	7	22	59.5	281
15–19	23	17.0 (16.2–17.7)	39.1	11	2	1	1	1	0	0	0	0	7	16	69.6	163
20+	71	31.6 (29.1–34)	46.5	20	3	0	1	0	9	2	1	1	34	24	33.8	103

[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.

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, nine of whom met the exclusion criteria at this time point and were excluded from longitudinal analysis. Age, gender, and baseline *P. falciparum* positivity did not differ significantly between the groups for whom a second venous sample was or was not available.

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, and ≥15 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 *P. 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

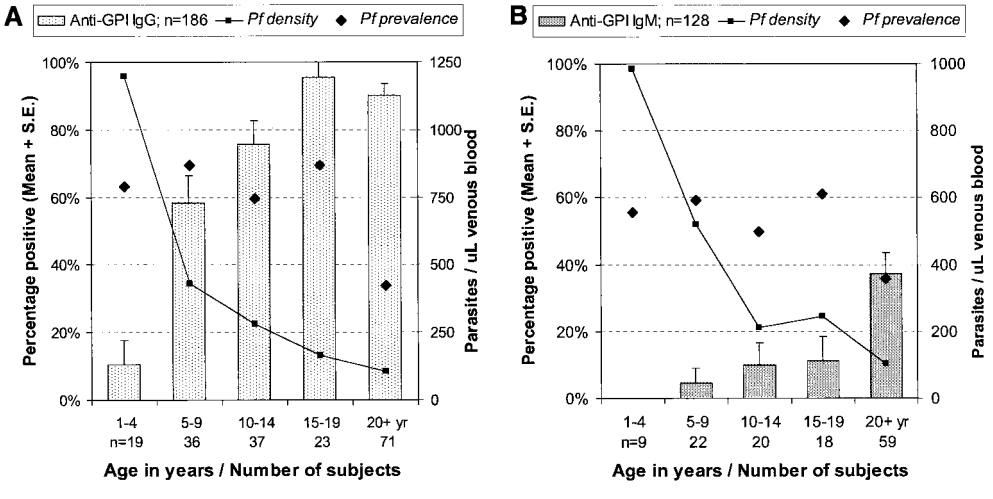

FIG. 1. Percentage of subjects positive for IgG antibodies to GPIs (means + standard errors [S.E.]) (A) and IgM antibodies (B). Diamonds represent the percentage of subjects with *P. falciparum* (*Pf*) parasitemia either alone or in combination with other parasites for each age group; squares represent the geometric mean density of parasitemia (solid line).

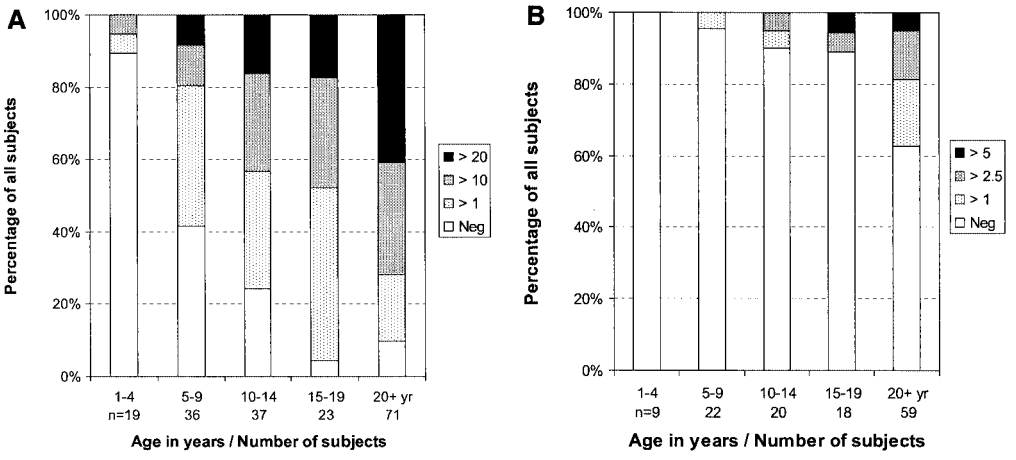

FIG. 2. Intensity of IgG antibody response to GPI (A) and IgM antibody responses (B). The upper limit (mean plus 2 standard deviations) of the responses in 15 non-malaria-exposed Australian adult controls was arbitrarily assigned a value of 1, and antibody responses in study subjects were expressed as a multiple of this value (units). Positive responses of increasing intensity are shown in progressively darker shades. Negative responses (Neg) represent adjusted absorbance readings less than the upper limit of the nonexposed controls (i.e., $\leq$ 1 U).

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 *P. falciparum* parasitemia (alone or in combination with other parasites) were IgM positive compared to 25% (9 of 36) without *P. falciparum* (including aparasitemic subjects [OR, 3.9; 95% CI, 1.3 to 11.9; $P = 0.015$; χ^2 test]). Excluding subjects with mixed infections, anti-GPI IgMs were present in 55.6% (10 of 18) of subjects aged ≥20 years with *P. falciparum* parasitemia, compared to 0 of 7 subjects with *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%).

Longitudinal antibody response to GPI following clearance of parasitemia. Paired antibody responses were examined in subjects whose baseline *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 *P. falciparum* was noted at follow-up. All 18 seronegative subjects with *P. falciparum* parasitemia at baseline remained seronegative at follow-up and were therefore not included in this analysis.

Eradication of *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 *P. falciparum* parasitemia at either time point, either overall (mean decrease, 3.6%; $P = 0.55$) or in age-based subgroups.

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 *P. falciparum* parasitemia. Eradication of asymptomatic *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 *P. falciparum* was associated with the presence of IgM antibodies to GPIs in subjects aged ≥20 years. Together these observations indicate that *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 *P. falciparum* and anti-GPI IgGs. Children and adolescents aged 4 to 14 years from Madang Province have frequent subpatent 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 *P. vivax* induces antibodies that are cross-reactive with *P. falciparum* GPIs (4, 6). In our study, IgM anti-GPI antibody responses were absent in subjects aged ≥20 years with *P. vivax* infection but were present in a majority of subjects with *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 likelier 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.

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REFERENCES

1. **Anstey, N. M., J. B. Weinberg, M. Y. Hassanali, E. D. Mwaikambo, D. Manyenga, M. A. Misukonis, D. R. Arnelle, D. Hollis, M. I. McDonald, and D. L. Granger.** 1996. Nitric oxide in Tanzanian children with malaria: inverse relationship between malaria severity and nitric oxide production/nitric oxide synthase type 2 expression. J. Exp. Med. **184:**557–567.
2. **Anstey, N. M., J. B. Weinberg, Z. Wang, E. D. Mwaikambo, P. E. Duffy, and D. L. Granger.** 1999. Effects of age and parasitemia on nitric oxide production/leukocyte nitric oxide synthase type 2 expression in asymptomatic, malaria-exposed children. Am. J. Trop. Med. Hyg. **61:**253–258.
3. **Bate, C. A., and D. Kwiatkowski.** 1994. A monoclonal antibody that recognizes phosphatidylinositol inhibits induction of tumor necrosis factor alpha by different strains of *Plasmodium falciparum*. Infect. Immun. **62:**5261–5266.
4. **Bate, C. A., and D. Kwiatkowski.** 1994. Inhibitory immunoglobulin M antibodies to tumor necrosis factor-inducing toxins in patients with malaria. Infect. Immun. **62:**3086–3091.
5. **Bate, C. A., J. Taverne, A. Dave, and J. H. Playfair.** 1990. Malaria exoantigens induce T-independent antibody that blocks their ability to induce TNF. Immunology **70:**315–320.
6. **Bate, C. A., J. Taverne, N. D. Karunaweera, K. N. Mendis, D. Kwiatkowski, and J. H. Playfair.** 1992. Serological relationship of tumor necrosis factor-inducing exoantigens of *Plasmodium falciparum* and *Plasmodium vivax*. Infect. Immun. **60:**1241–1243.
7. **Bruce, M. C., C. A. Donnelly, M. P. Alpers, M. R. Galinski, J. W. Barnwell, D. Walliker, and K. P. Day.** 2000. Cross-species interactions between malaria parasites in humans. Science **287:**845–848.
8. **Bruce, M. C., C. A. Donnelly, M. Packer, M. Lagog, N. Gibson, A. Narara, D. Walliker, M. P. Alpers, and K. P. Day.** 2000. Age- and species-specific duration of infection in asymptomatic malaria infections in Papua New Guinea. Parasitology **121:**247–256.
9. **Bruce, M. C., M. R. Galinski, J. W. Barnwell, C. A. Donnelly, M. Walmsley, M. P. Alpers, D. Walliker, and K. P. Day.** 2000. Genetic diversity and dynamics of Plasmodium falciparum and P. vivax populations in multiply infected children with asymptomatic malaria infections in Papua New Guinea. Parasitology **121:**257–272.
10. **Burkot, T. R., P. M. Graves, J. A. Cattani, R. A. Wirtz, and F. D. Gibson.** 1987. The efficiency of sporozoite transmission in the human malarias, Plasmodium falciparum and P. vivax. Bull. W. H. O. **65:**375–380.

11. **Burkot, T. R., P. M. Graves, R. Paru, R. A. Wirtz, and P. F. Heywood.** 1988. Human malaria transmission studies in the Anopheles punctulatus complex in Papua New Guinea: sporozoite rates, inoculation rates, and sporozoite densities. Am. J. Trop. Med. Hyg. **39:**135–144.
12. **Cattani, J. A., J. L. Tulloch, H. Vrbova, D. Jolley, F. D. Gibson, J. S. Moir, P. F. Heywood, M. P. Alpers, A. Stevenson, and R. Clancy.** 1986. The epidemiology of malaria in a population surrounding Madang, Papua New Guinea. Am. J. Trop. Med. Hyg. **35:**3–15.
13. **Charlwood, D.** 1999. Malaria drug trials—an ethical dilemma? Parasitol. Today **15:**346.
14. **Clark, I. A., F. M. al-Yaman, W. B. Cowden, and K. A. Rockett.** 1996. Does malarial tolerance, through nitric oxide, explain the low incidence of autoimmune disease in tropical Africa? Lancet **348:**1492–1494.
15. **Clark, I. A., and L. Schofield.** 2000. Pathogenesis of malaria. Parasitol. Today **16:**451–454.
16. **Cochran, W. G.** 1954. Some methods for strengthening the common X^2 tests. Biometrics **10:**417–451.
17. **Cox, M. J., D. E. Kum, L. Tavul, A. Narara, A. Raiko, M. Baisor, M. P. Alpers, G. F. Medley, and K. P. Day.** 1994. Dynamics of malaria parasitaemia associated with febrile illness in children from a rural area of Madang, Papua New Guinea. Trans. R. Soc. Trop. Med. Hyg. **88:**191–197.
18. **Delley, V., P. Bouvier, N. Breslow, O. Doumbo, I. Sagara, M. Diakite, A. Mauris, A. Dolo, and A. Rougemont.** 2000. What does a single determination of malaria parasite density mean? A longitudinal survey in Mali. Trop. Med. Int. Health **5:**404–412.
19. **Druilhe, P., and J. L. Perignon.** 1999. Malaria from Africa blows hot and cold. Nat. Med. **5:**272–273.
20. **Gupta, S., and K. P. Day.** 1994. A theoretical framework for the immunoepidemiology of Plasmodium falciparum malaria. Parasite Immunol. **16:**361–370.
21. **Janeway, C. A., P. Travers, S. Hunt, and M. Walport.** 1997. Immunobiology: the immune system in health and disease, 3rd ed. Current Biology Ltd., London, United Kingdom.
22. **King, M.** 1973. A medical laboratory for developing countries. Oxford University Press, London, United Kingdom.
23. **Kwiatkowski, D.** 1995. Malarial toxins and the regulation of parasite density. Parasitol. Today **11:**206–212.
24. **Marsh, K., and R. W. Snow.** 1997. Host-parasite interaction and morbidity in malaria endemic areas. Philos. Trans. R. Soc. Lond. B Biol. Sci. **352:**1385–1394.
25. **Mehlotra, R. K., K. Lorry, W. Kastens, S. M. Miller, M. P. Alpers, M. Bockarie, J. W. Kazura, and P. A. Zimmerman.** 2000. Random distribution of mixed species malaria infections in Papua New Guinea. Am. J. Trop. Med. Hyg. **62:**225–231.
26. **Miller, M. J.** 1958. Observations of the natural history of malaria in the semi-resistant west African. Trans. R. Soc. Trop. Med. Hyg. **52:**152–168.
27. **Moingeon, P., J. Haensler, and A. Lindberg.** 2001. Towards the rational design of Th1 adjuvants. Vaccine **19:**4363–4372.
28. **Molano, A., S. H. Park, Y. H. Chiu, S. Nosseir, A. Bendelac, and M. Tsuji.** 2000. Cutting edge: the IgG response to the circumsporozoite protein is MHC class II-dependent and CD1d-independent: exploring the role of GPIs in NK T cell activation and antimalarial responses. J. Immunol. **164:**5005–5009.
29. **Naik, R. S., O. H. Branch, A. S. Woods, M. Vijaykumar, D. J. Perkins, B. L. Nahlen, A. A. Lal, R. J. Cotter, C. E. Costello, C. F. Ockenhouse, E. A. Davidson, and D. C. Gowda.** 2000. Glycosylphosphatidylinositol anchors of Plasmodium falciparum: molecular characterization and naturally elicited antibody response that may provide immunity to malaria pathogenesis. J. Exp. Med. **192:**1563–1576.
30. **Playfair, J. H., J. Taverne, C. A. Bate, and J. B. de Souza.** 1990. The malaria vaccine: anti-parasite or anti-disease? Immunol. Today **11:**25–27.
31. **Porcelli, S. A., and R. L. Modlin.** 1999. The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. Annu. Rev. Immunol. **17:**297–329.
32. **Rogier, C., D. Commenges, and J. F. Trape.** 1996. Evidence for an age-dependent pyrogenic threshold of Plasmodium falciparum parasitemia in highly endemic populations. Am. J. Trop. Med. Hyg. **54:**613–619.
33. **Rogier, C., A. Tall, N. Diagne, D. Fontenille, A. Spiegel, and J. F. Trape.** 1999. Plasmodium falciparum clinical malaria: lessons from longitudinal studies in Senegal. Parassitologia **41:**255–259.
34. **Schofield, L., and F. Hackett.** 1993. Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites. J. Exp. Med. **177:**145–153.
35. **Schofield, L., M. J. McConville, D. Hansen, A. S. Campbell, B. Fraser-Reid, M. J. Grusby, and S. D. Tachado.** 1999. CD1d-restricted immunoglobulin G formation to GPI-anchored antigens mediated by NKT cells. Science **283:**225–229.
36. **Schofield, L., S. Novakovic, P. Gerold, R. T. Schwarz, M. J. McConville, and S. D. Tachado.** 1996. Glycosylphosphatidylinositol toxin of Plasmodium upregulates intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin expression in vascular endothelial cells and increases leukocyte and parasite cytoadherence via tyrosine kinase-dependent signal transduction. J. Immunol. **156:**1886–1896.
37. **Schofield, L., L. Vivas, F. Hackett, P. Gerold, R. T. Schwarz, and S. Tachado.** 1993. Neutralizing monoclonal antibodies to glycosylphosphatidylinositol, the dominant TNF-alpha-inducing toxin of Plasmodium falciparum: prospects for the immunotherapy of severe malaria. Ann. Trop. Med. Parasitol. **87:**617–626.
38. **Sieling, P. A., D. Chatterjee, S. A. Porcelli, T. I. Prigozy, R. J. Mazzaccaro, T. Soriano, B. R. Bloom, M. B. Brenner, M. Kronenberg, P. J. Brennan, et al.** 1995. CD1-restricted T cell recognition of microbial lipoglycan antigens. Science **269:**227–230.
39. **Sinton, J. A.** 1938. Immunity or tolerance in malarial infections. Proc. R. Soc. Med. **31:**1298–1302.
40. **Smith, T., B. Genton, K. Baea, N. Gibson, J. Taime, A. Narara, F. Al-Yaman, H. P. Beck, J. Hii, and M. Alpers.** 1994. Relationships between Plasmodium falciparum infection and morbidity in a highly endemic area. Parasitology **109:**539–549.
41. **Snow, R. W., and K. Marsh.** 1998. New insights into the epidemiology of malaria relevant for disease control. Br. Med. Bull. **54:**293–309.
42. **Tachado, S. D., P. Gerold, M. J. McConville, T. Baldwin, D. Quilici, R. T. Schwarz, and L. Schofield.** 1996. Glycosylphosphatidylinositol toxin of Plasmodium induces nitric oxide synthase expression in macrophages and vascular endothelial cells by a protein tyrosine kinase-dependent and protein kinase C-dependent signaling pathway. J. Immunol. **156:**1897–1907.
43. **Taylor-Robinson, A.** 2001. Rationale for malaria anti-toxin therapy. Trends Parasitol. **17:**119.
44. **Taylor-Robinson, A. W., and E. C. Smith.** 1999. A role for cytokines in potentiation of malaria vaccines through immunological modulation of blood stage infection. Immunol. Rev. **171:**105–123.
45. **Trape, J. F., P. Peelman, and B. Morault-Peelman.** 1985. Criteria for diagnosing clinical malaria among a semi-immune population exposed to intense and perennial transmission. Trans. R. Soc. Trop. Med. Hyg. **79:**435–442.

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