Glycosylphosphatidylinositol Anchors of *Plasmodium falciparum*: Molecular Characterization and Naturally Elicited Antibody Response That May Provide Immunity to Malaria Pathogenesis

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Abstract

Induction of proinflammatory cytokine responses by glycosylphosphatidylinositols (GPIs) of intraerythrocytic *Plasmodium falciparum* is believed to contribute to malaria pathogenesis. In this study, we purified the GPIs of *P. falciparum* to homogeneity and determined their structures by biochemical degradations and mass spectrometry. The parasite GPIs differ from those of the host in that they contain palmitic (major) and myristic (minor) acids at C-2 of inositol, predominantly C18:0 and C18:1 at *sn*-1 and *sn*-2, respectively, and do not contain additional phosphoethanolamine substitution in their core glycan structures. The purified parasite GPIs can induce tumor necrosis factor α release from macrophages. We also report a new finding that adults who have resistance to clinical malaria contain high levels of persistent anti–GPI antibodies, whereas susceptible children lack or have low levels of short-lived antibody response. Individuals who were not exposed to the malaria parasite completely lack anti–GPI antibodies. Absence of a persistent anti–GPI antibody response correlated with malaria-specific anemia and fever, suggesting that anti–GPI antibodies provide protection against clinical malaria. The antibodies are mainly directed against the acylated phosphoinositol portion of GPIs. These results are likely to be valuable in studies aimed at the evaluation of chemically defined structures for toxicity versus immunogenicity with implications for the development of GPI-based therapies or vaccines.

Key words: malaria parasite • cytokine response • antigenicity • acquired immunity • pathogenesis

Introduction

The rapid spread of drug-resistant *Plasmodium falciparum* and the lack of an effective malaria vaccine present an urgent need for alternative approaches to prevent malaria infection and pathogenesis (1). It is becoming increasingly clear that complete protection against malaria infection and illness requires multifactorial immunity (2). Currently,

most vaccine efforts are aimed at immunity against infection (antiparasitic) by targeting the parasite proteins (2–5). However, it has long been suggested that immunity against severe malaria is partly antiparasitic and partly antitoxic (toxic effects in response to parasite factors). The majority of the adults in malaria endemic areas have resistance to severe malaria. However, most children <4 yr of age are susceptible despite exposure to high malaria transmission, which can produce high levels of antibodies against protein antigens including merozoite surface protein (MSP)[1]-1, erythrocyte membrane antigen (EBA)-175, and apical membrane antigen 1 (AMA)-1. Although antibody responses against parasite proteins correlate with protection against parasitemia (Branch, O.H., unpublished results), resistance to malaria illness is independent of parasitemia levels. This agrees with the resistance of adults and older children to malaria pathology even though they can develop significant parasitemia (6); conversely, severe illness can occur at relatively low-density parasitemias independent of antibody response against parasite proteins (7–9). The factors associated with the resistance to clinical disease (antidisease immunity) have not been established; understanding these would lead to alternative approaches for malaria control. In this regard, parasite glycosylphosphatidylinositols (GPIs) appear to offer new opportunities.

GPIs are a distinct class of glycolipids found ubiquitously in eukaryotic cells and implicated in several biological responses (10–12). GPIs are particularly abundant in parasites, where they are found as free lipids and attached to proteins. In intraerythrocytic *P. falciparum*, GPIs represent the major glycoconjugates. Several functionally important parasite proteins, including MSP-1, MSP-2, and MSP-4, are anchored to the cell membranes through GPI moieties (13–17). Recently, we have shown that *P. falciparum* synthesizes GPIs in a developmental stage–specific manner and that GPI biosynthesis is crucial for the development and survival of the parasite (18). The enzyme specificity of some key steps of parasite GPI biosynthesis differs significantly from those of the host, suggesting the possibility of targeting the parasite GPI structures for the development of antiparasitic drugs. However, detailed structures of parasite GPIs have not been determined. Although the structures of glycan cores have been established using metabolically labeled GPIs (19, 20), details regarding the nature of various acyl residues and other possible substitutents were not clear (21). Determination of a detailed structure requires isolation of pure GPIs which, in the case of *P. falciparum*, is a challenge due to the difficulty in obtaining adequate amounts of parasites free of host cell components. In this study, we were able to successfully purify *P. falciparum* GPIs to homogeneity and establish their structures.

It has long been believed that malaria pathology is due to factors endogenously produced in response to parasite toxins. Several studies have shown that malaria pathology is at least in part due to parasite toxic factors that can induce TNF-α and other cytokines, which could then lead to clinical effects including fever, hypoglycemia, dyserythropoiesis, and vascular damage in the lungs and brain (22, 23). This agrees with the elevated levels of TNF-α in patients with lethal cerebral malaria (24) and the ability of anti–TNF-α antibodies to prevent lethal cerebral pathology in mice (25).

P. falciparum GPIs have been identified as malaria pathogenicity factors based on their ability to induce inflammatory cytokines in macrophages and endothelial cells and cause symptoms reminiscent of acute malaria infection in experimental animals (26–29). Schofield et al. (26) have shown that parasite fractions enriched with GPIs can induce TNF-α and IL-1 in macrophages; in mice, GPIs can cause transient pyrexia, hypoglycemia, lethal cachexia, and even death in D-galactosamine (GalN)-sensitized animals. Schofield et al. have also shown that *P. falciparum* GPIs exert toxic effects through the expression of TNF-α, IL-1, inducible nitric oxide synthase (iNOS), and endothelial cell adhesion molecules by activating nuclear factor κB transcription factors (27–29). As *P. falciparum*–infected erythrocytes are sequestered in specific organs, the local high concentrations of toxic responses to the parasite GPIs can affect vital physiologic functions and cause severe illness. Recent studies have shown that GPIs from a *Trypanosoma cruzi* mucin can also induce proinflammatory cytokines (30). The antagonists of GPI-mediated signaling and a monoclonal antibody against *P. falciparum* GPIs can block the induction of toxic responses (27–29), suggesting that GPI-based therapy is possible.

Because *P. falciparum* GPIs are pathogenicity factors, we hypothesized that adults in malaria endemic areas should have GPI-specific protective immunity. We tested this hypothesis by analyzing the anti-GPI antibody response in sera from a longitudinal cohort study and in sera of a large group of adults from Western Kenya. The data demonstrate for the first time that people living in malaria endemic areas elicit a parasite GPI–specific IgG response in an age-dependent manner; although adults and older children have high levels of antibodies, malaria-susceptible children either lack or have only very low levels of short-lived antibodies. The results also suggest the involvement of anti-GPI antibodies in protection against malaria pathogenesis.

Materials and Methods

Reagents. Human blood and serum were purchased from Interstate Blood Bank. RPMI 1640, DME, and cell culture reagents were from Life Technologies. Gelatin, bee venom phospholipase A$_2$ (1,800 U/mg), standard phospholipids, and saponin were from Sigma-Aldrich. Silica Gel 60 high performance thin-layer chromatography (HPTLC) plates were from either EM Science or Whatman. Pronase was purchased from Calbiochem. *Aspergillus saitoi* α-mannosidase (400 mU/mg) and jack bean α-mannosidase

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[1]*Abbreviations used in this paper:* AHM, 2,5-anhydromannitol; CL, cardiolipin; EBA, erythrocyte membrane antigen; GalN, galactosamine; GC-MS, gas chromatography–mass spectrometry; GlcN, glucosamine; GPI, glycosylphosphatidylinositol; HF, hydrofluoric acid; HPTLC, high performance thin-layer chromatography; HRP, horseradish peroxidase; MSP, merozoite surface protein; PG, phosphoglyceride; PI, phosphatidylinositol.

(30 U/mg) were from Oxford Glycosystems. Poly(isobutyl methacrylate) was procured from Polysciences, Inc. Horseradish peroxidase (HRP)-conjugated goat anti–human IgG (H and L chains) and 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) reagent were from Kirkegaard & Perry Laboratories. Microtiter plates were from Dynex Technologies. [6-^{3}H]glucosamine (GlcN; 23 Ci/mmol), and [^{3}H]arachidonic (202 Ci/mmol), [^{3}H]myristic (55 Ci/mmol), and [^{3}H]oleic acids (8 Ci/mmol) were from Amersham Pharmacia Biotech. [^{3}H]Linoleic (60 Ci/mmol), [^{3}H]palmitic (60 Ci/mmol), [^{3}H]palmitoleic (60 Ci/mmol), and [^{3}H]stearic acids (60 Ci/mmol) were from American Radiolabeled Chemicals. ^{125}I-labeled goat anti–human IgG (8.66 μCi/μg) and En^{3}Hance were from NEN Life Science Products. Murine macrophages (J774A.1) were from American Type Culture Collection. HPLC grade solvents were used throughout the study.

Cell Culture and Isolation of Parasites. Intraerythrocytic *P. falciparum* (FCR-3 strain) was cultured in RPMI 1640 using human O-type erythrocytes and 10% O-positive human serum at 3–4% hematocrit (18, 19). Cultures were routinely synchronized with 5% sorbitol (31) and tested for mycoplasma (32); only contamination-free cultures were used.

Cultures with 20–30% parasitemia were harvested at midschizont stage. Packed erythrocytes (20 ml) suspended in 180 ml of 0.65% gelatin in culture medium were incubated at 37°C for 30 min (33). The enriched infected erythrocytes (70–80% parasitemia) in the supernatant were recovered and lysed with 0.015% saponin in Trager's buffer (34). The suspension was passed through a 26-gauge needle to solubilize the erythrocyte debris and then centrifuged. The parasites were suspended in the above buffer, layered on 5% BSA, and centrifuged (35). The packed parasites (∼4 ml) were washed with the buffer and stored at −80°C until used.

Metabolic Labeling of the GPIs. Parasites were radiolabeled in medium containing 5 mM glucose and [^{3}H]GlcN (50 μCi/ml; reference 19). Labeling with ^{3}H–fatty acids (50 μCi/ml) was performed in medium containing 2% serum and 20 mM glucose.

Isolation of GPIs. All procedures were carried out using acid-washed, siliconized glassware to prevent surface adsorption of GPIs, high quality solvents, and sterile water and buffers. Parasites (10 ml) were lyophilized and extracted three times with 50 ml of chloroform/methanol (2:1, vol/vol) to remove nonglycosylated lipids. The pellet was extracted five times with 50 ml of CMW (chloroform/methanol/water, 10:10:3 [vol/vol/vol]), dried, and then partitioned between water and water-saturated 1-butanol. The organic layer was dried and the residue was extracted with 80% aqueous 1-propanol. Finally, GPIs were purified by HPLC and HPTLC.

To isolate GPIs that are linked to parasite proteins, the delipidated parasite pellet was digested with pronase (50 U/ml) in 50 ml of 100 mM NH$_4$HCO$_3$, 1 mM CaCl$_2$, pH 8.0, at 37°C for 24 h. The released GPIs (designated as amino acid–linked GPIs) were extracted with water-saturated 1-butanol, washed with water, dried, and purified by HPLC. Control erythrocyte membrane debris, saponin-lysate, and ghosts were similarly extracted and fractionated.

HPLC Purification of GPIs. The GPIs (∼10 μg plus 400,000 cpm of [^{3}H]GlcN-labeled GPIs) were chromatographed on a C$_4$ reversed phase Supelcosil LC-304 HPLC column (4.6 × 250 mm, 5 μm particle size; Supelco) using a linear gradient of 20–60% aqueous 1-propanol containing 0.1% TFA over a period of 80 min and held for 30 min at a flow rate of 0.5 ml/min (36). Fractions (1.0 ml) were collected, and elution of GPIs was moni-

tored by measuring radioactivity. Aliquots (0.5 μl) were also assayed by ELISA with Kenyan adult sera. Glycolipids extracted from control erythrocyte membrane debris, total lysate, and ghosts were similarly chromatographed, and fractions were analyzed for reactivity with Kenyan sera.

Purification and Analysis of GPIs by HPTLC. The HPLC-purified GPIs (5 μg) were applied onto 10 × 10–cm plates as continuous streaks. Parallel spots with [^{3}H]GlcN-labeled GPIs (50,000 cpm) were used for monitoring GPI bands by fluorography using En^{3}Hance (18, 19). The plates were developed with CMW (10:10:2.5, vol/vol/vol). GPIs from the plates were extracted with CMW (10:10:3, vol/vol/vol), dried, dissolved in water-saturated 1-butanol, and washed with water.

In separate experiments, HPTLC plates were scraped (0.5-cm-width fractions), and GPIs were extracted and analyzed by ELISA using Kenyan adult sera. Glycolipids from control erythrocyte membrane debris, total lysate, and ghosts were similarly analyzed.

Compositional Analysis. HPLC- and HPTLC-purified, free and amino acid–linked GPIs (4–5 μg each), and hydrofluoric acid (HF)-released inositol-acylated carbohydrate moiety (2–3 μg), and diacylglycerol moiety (1–2 μg) were treated with 8 M anhydrous methanolic KOH for 2 h at 120°C. After acidification with cold dilute HCl, the fatty acid methyl esters were extracted with chloroform. Phospholipase A$_2$–released fatty acids were esterified with methyl iodide in DMSO/solid NaOH. Gas chromatography–mass spectrometry (GC-MS) was performed on a ThermoQuest GCQ^{plus} ion trap electron ionization quadrupole mass spectrometer (Finnigan) using DB-5 capillary column (30 m × 0.25 mm interior diameter with 0.25-μm film; Alltech). The temperature was held at 75°C for 1 min, raised to 150°C at 1°C/min, and then to 325°C at 8°C/min. GC retention times and mass spectra were compared against standards.

The free and amino acid–linked GPIs (∼2 μg each) were hydrolyzed with 2.5 M TFA at 100°C for 5 h, and the hydrolysates were analyzed for mannose by HPLC (37).

Mass Spectrometry. Matrix-assisted laser-desorption/ionization time of flight mass spectrometry analysis was performed using a Kratos analytical MALDI-4 mass spectrometer equipped with a nitrogen laser at 20-kV accelerating voltage (38). Spectra were acquired with a time-delayed extraction and were the average of 50 laser shots. The matrix was saturated with α-cyano-4-hydroxycinnamic acid in 50% ethanol. The mass accuracy was within 2 daltons.

TNF-α Induction by GPIs. Murine macrophages (1.5 × 10^6 cells/well in 96-well plates) in DMEM and 10% fetal bovine serum were stimulated with 0.03–0.5 μM HPTLC-purified, free and amino acid–linked GPIs (see Fig. 1 B, lanes 2 and 4, respectively). TNF-α in the culture supernatants was measured by a quantitative sandwich ELISA using the TNF-α estimation kit (R&D Systems). Experiments were performed in triplicate with three separate batches of GPI preparations.

IgG-specific ELISA. HPLC-purified, free GPIs dissolved in methanol were coated (0.25–32 ng/well) onto 96-well microtiter plates. After evaporation of methanol at 37°C, the wells were blocked with 0.5% casein in 50 mM Tris-buffered saline, pH 7.4 (TBS-casein), and incubated with serially diluted sera (1:100 to 1:64,000) in TBS-casein containing 0.05% Tween 20. Initially, randomly selected 20 Kenyan and 2 USA adult sera were assayed. The bound antibodies were measured by HRP-conjugated goat anti–human IgG and ABTS substrate. All other sera were analyzed at 1:200 or 1:400 dilution by coating GPIs at 0.5–2 ng/well.

Competitive Inhibition ELISA. Sera were diluted 1:200 in TBS-casein containing 0.05% Tween 20 and incubated at room

temperature for 30 min with various concentrations of phosphatidylinositols (PIs), phosphoglyceride (PG), cardiolipin (CL), or GPI, and then ELISA was performed as described above.

TLC Immunoblotting. The HPLC-purified, free and amino acid–linked GPIs (100 ng each) were chromatographed on HPTLC plates. The plates were soaked in 0.1% poly(isobutyl methacrylate) in hexane, dried, and incubated for 2 h at room temperature with TBS containing 1% BSA and then for 2 h in 1:100 diluted sera (39). The plates were washed with cold phosphate-buffered saline, incubated with ^{125}I-labeled goat anti–human IgG (5 μCi/ml) for 1 h, washed, dried, and exposed to x-ray film.

Nitrous Acid Treatment. The HPLC-purified, free GPIs (20 μg) suspended in 150 μl 0.2 M NaOAc, pH 3.8, were treated with 150 μl 1 M NaNO$_2$ at room temperature for 24 h (40). The released PI moieties were extracted with water-saturated 1-butanol. The glycan moieties were recovered by chromatography on a Bio-Gel P-4 column (1 × 90 cm) in 100 mM pyridine, 100 mM HOAc, pH 5.2. These were used to assess seroreactivity.

Treatment with HF. The HPLC-purified, free GPIs (2 μg plus 500,000 cpm of [3H]GlcN-labeled GPIs) were treated with 50% aqueous HF (50 μl) in an ice bath for 48 h (40). The reaction mixture was neutralized with LiOH and extracted with water-saturated 1-butanol. The organic layer was washed with water, dried, and the carbohydrate moiety was purified by HPLC.

Treatment with Mannosidase. The [3H]GlcN-labeled GPI glycan cores (10,000 cpm) were digested with jack bean α-mannosidase and the products were deionized with AG 50W-X16 (H$^+$) and lyophilized (19). The glycan cores (5,000 cpm) were digested with *A. saitoi* α-mannosidase (0.5 mU/ml), deionized, and lyophilized (19).

Treatment with Phospholipase A$_2$. HPLC-purified GPIs (2 μg) in 100 μl 100 mM Tris-HCl, 10 mM CaCl$_2$, pH 7.5, were treated with bee venom phospholipase A$_2$ (2,400 U/ml) at 37°C for 18 h. The digest was extracted with water-saturated 1-butanol and purified by HPLC; the retention time of *lyso*-GPIs is 75.5 min.

Analysis of Cohort Sera for Anti-GPI Antibodies. Sera were obtained from subjects of a longitudinal malaria project performed in a holoendemic, rural region of Western Kenya within the context of the Asembo Bay Cohort Project (ABCP [8]). Studies done in the subjects' households show that from March to May the entomological inoculation rate (EIR) was ∼30 infected bites per person per month, whereas during the drier months the EIR was ∼4. Pregnant mothers were identified and, after written consent, they, their delivered infants, and older children living in the same household were enrolled. The mothers and children were followed every 2 wk for ∼4 yr, and clinical parameters, axillary temperature, and illness were recorded. Blood samples were taken monthly and parasite and hemoglobin density were measured. More than 90% of detected parasitemias contained *P. falciparum* species.

Sera of 100 7–8-yr-old children and 50 nonpregnant adults, collected during February through August 1993, were analyzed at two time points, spaced 1–3 mo apart. For data analysis, we selected two time points for the young children, in the same manner as the older individuals, when they were ∼0.5, 1.5, 2.5, and 3.5 yr old. The study was approved by the Centers for Disease Control and Prevention and Kenyan Institutional Review Boards.

Analysis of the Relationship between Serum Anti-GPI Antibodies and Resistance to Malaria Pathogenesis. Analysis for correlation of anti-GPI antibody response with either hemoglobin or tempera-

ture as the dependent variable was done with the general linear model, Generalized Estimating Equations (41). The variables considered in the analyses were (a) anti–GPI antibody responder category (positive, intermittent, or negative), (b) parasite density, $\log_{10}(n + 1/\mu l)$, (c) age, and (d) antimalaria drug treatment. We controlled for the repeated measures on each patient. The estimated effect of positive and negative antibody responder category are given relative to that of the intermittent antibody responder category.

Results

Isolation and Purification of GPIs from Intraerythrocytic P. falciparum. As contamination with glycolipids from host erythrocytes and mycoplasma is a major concern in isolating GPIs from intraerythrocytic *P. falciparum*, we used rigorous protocols. First, parasite cultures were routinely tested for mycoplasma, and parasite-infected erythrocytes were enriched from cultures with 25–30 to 70–80% parasitemia to minimize erythrocyte components. Second, the parasites were released from infected erythrocytes by mild saponin lysis, washed, and purified by density centrifugation to remove erythrocyte membrane debris; metabolically [3H]GlcN-labeled cultures revealed that this procedure did not release parasite GPIs. These procedures eliminated all of the erythrocyte components. Third, the purified parasites were differentially extracted to remove most nonglycosylated lipids, and the extract containing the free GPIs (not linked to proteins) was subjected to solvent fractionation to eliminate soluble hemozoin and other colored components. Finally, the GPIs were purified by successive fractionation using HPLC and HPTLC. The GPIs that are linked to parasite proteins were isolated after exhaustive digestion of the delipidated parasites with pronase, and are referred to as amino acid–linked GPIs. The yields of the HPLC-purified free and amino acid–linked GPIs were 100–120 and 25–30 μg per 10 ml of packed purified parasites, respectively, based on the mannose content.

The parasite GPIs were purified by HPLC using a C$_4$ reversed phase column, which separated matured GPIs from GPI intermediates (Fig. 1 A). The elution of GPIs was monitored by radioactivity, which overlapped with the reactivity of the GPIs to human sera containing anti–GPI antibodies (Fig. 1 A). The compounds isolated from the extracts of erythrocyte debris, lysates, and ghosts were fractionated in parallel with the purification of parasite GPIs by HPLC (Fig. 1 A) and HPTLC and analyzed as controls. These did not react with Kenyan adult sera (Fig. 1 A), suggesting that the activity is specific to parasite GPIs.

The GPIs were further purified by HPTLC; they migrated as broad major bands and in some instances separated into two major bands (Fig. 1 B). The heterogeneity of GPIs is due to the variation in the fatty acid composition of individual GPIs (see below). The HPTLC-purified GPIs (Fig. 1 B, lanes 2 and 4) contained mannose and GlcN in molar ratios of ∼4:1 and were susceptible to nitrous acid, HF, and alkali (not shown). Fatty acid compositional analysis of HPLC-purified GPIs showed the presence of myristic
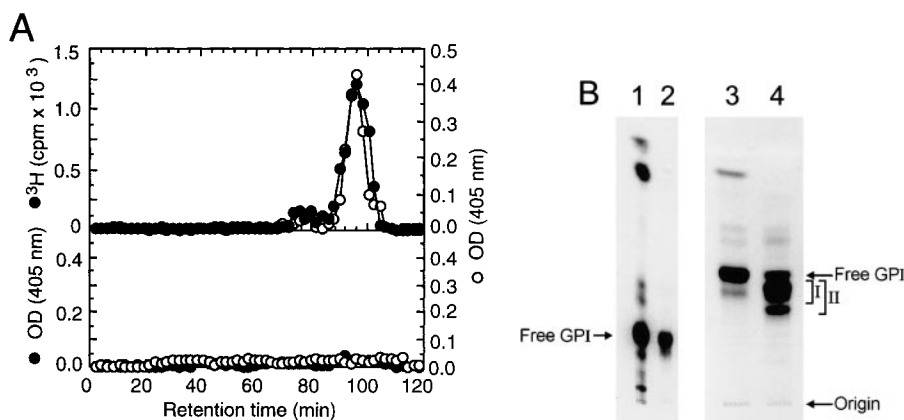
Figure 1. HPLC and HPTLC purification of *P. falciparum* GPIs. (A) The parasite GPIs (10 μg plus 400,000 cpm of [³H]GlcN-labeled GPIs) were chromatographed on a 4.6 × 250–mm C_4 reversed phase HPLC column with a linear gradient of 20–60% aqueous 1-propanol (reference 37). (Top) Analysis of parasite GPIs: fractions (1.0 ml) were collected and ³H activity in 5-μl aliquots was measured (●); 0.5-μl aliquots assayed by ELISA for immunoreactivity with Kenyan adult sera (○). (Bottom) Analysis of glycolipids from control erythrocyte membrane debris obtained from 4 ml packed erythrocytes (●), and those of delipidated, pronase-digested erythrocyte ghosts from 2 ml packed erythrocytes (○). 1-μl aliquots were assayed for immunoreactivity with Kenyan sera. Extracts of total erythrocyte lysate were similarly analyzed (not shown). (B) Fluorograms of the [³H]GlcN-labeled GPIs chromatographed on Silica Gel 60 HPTLC plates using CMW (10:10:2.5, vol/vol/vol). Lane 1, total free GPIs before HPLC; lane 2, HPLC-purified matured free GPIs; lane 3, total free GPIs (different preparation from that in lane 1; obtained by culturing parasites in regular medium after replacing medium with radiolabeled precursor to maximally convert intermediates into matured GPIs); and lane 4, HPLC-purified, amino acid–linked GPIs. Each lane contains 200 ng of GPIs plus 20,000 cpm of [³H]GlcN-labeled GPIs. Note that a small amount of free GPIs that remained with parasite pellet even after exhaustive extraction with organic solvents was copurified with amino acid–linked GPIs (lane 4).

(~4%), palmitic (~33%), stearic (~26%), oleic (~28%), cis-vaccenic (~2%), linoleic (~3%), arachidic (~2%), and behenic (~1%) acids, and 1–2% of unidentified acids. The presence of cis-vaccenic acid in the GPIs agrees with the ability of *P. falciparum* to synthesize two isomeric C18:1 acids (oleic and cis-vaccenic acids [42]).

Structural Analysis of P. falciparum GPIs. The structures of the purified *P. falciparum* GPIs were determined by specific chemical and enzymatic degradation studies using [³H]GlcN- and ³H–fatty acid-labeled GPIs and by mass spectrometry.

The [³H]GlcN-labeled GPIs were characterized by subjecting them to various degradative procedures. Treatment with jack bean α-mannosidase quantitatively converted the [³H]GlcN-labeled GPIs into species with three mannose residues, similar to the results recently reported (18), suggesting that the distal fourth mannose residue does not contain any substituent. The [³H]GlcN-labeled GPIs were dephosphorylated with HF and then deaminated and reduced with $NaBH_4$. The neutral glycan core, thus obtained, on HPTLC migrated as a single band with a mobility relative to the solvent front (R_f) value identical to previously characterized Man₄-2,5-anhydromannitol (AHM) (19). Treatment of the glycan core with *A. saitoi* α-mannosidase shifted the R_f value to that of authentic Man₂-AHM, and the product of jack bean α-mannosidase digestion comigrated with authentic AHM (not shown; 19). These results are consistent with the previously established structure of Manα1-2Manα1-2Manα1-6Manα1-4GlcN for the glycan core of *P. falciparum* GPIs (19, 20).

The mass spectrum of the purified, free GPIs (upper half portion of the HPTLC band in Fig. 1 B, lane 2) contained prominent molecular ions (M-H)⁻ at *m/z* 2,006.3 and 2,034.3 and minor ions at *m/z* 1,978.3, 2,062.3, and 2,090.3 (Fig. 2 A). The observed difference of 28 mass units (in some fractions 26 units) between the consecutive molecular ions suggests that the GPIs contain mixtures of homologous fatty acids differing by two carbons. The proportions of the GPI species varied considerably depending on the regions of HPTLC band analyzed (compare Fig. 2, A and B), suggesting heterogeneity with respect to acyl substitutents. Mass spectrometry of the control materials did not show molecular ions comparable to the parasite GPIs (not shown). These results show that the purified compounds are GPIs of the parasite, not of erythrocytes.

Treatment of free GPIs with phospholipase A_2 resulted in the marked loss of (M-H)⁻ ions of intact GPIs and the appearance of a new set of ions, each 264.5 mass units lower (Fig. 2 B), indicating that the GPIs contain a C18:1 acyl substituent at the *sn*-2 position. The spectra of some GPI fractions treated with phospholipase A_2 also contained significant levels of ions 262.5 mass units lower than the parent ions, suggesting the presence of GPIs with a C18:2 acid at *sn*-2. GC-MS analysis of phospholipase A_2–released fatty acids demonstrated the presence of predominantly oleic acid with minor amounts of cis-vaccenic and linoleic acids. These data together with the total fatty acid composition indicate that the parasite GPIs contain mainly oleic (~85%), cis-vaccenic (~6%), and linoleic (~9%) acids at *sn*-2.

As reported previously (21), the GPIs were sensitive to GPI-specific phospholipase D, but completely resistant to PI–phospholipase C (data not shown), suggesting the presence of an acyl substituent on C-2 of inositol. To identify this substituent, the GPIs were treated with HF and the released inositol-acylated carbohydrate moiety was purified by HPLC as a single symmetrical peak with a retention time of 47 min. Fatty acid compositional analysis indicated that the HF-released inositol-acylated carbohydrate moiety contained palmitic acid (~90%) and myritsic acid (~10%). Positive ion–mode mass spectrum showed (M+Na⁺) ions at *m/z* 1,224.4 and 1,252.7 (Fig. 2 C), which were assigned to Man₄-GlcN-inositol with myristoyl and palmitoyl substitution, respectively, on inositol. The signals at *m/z*
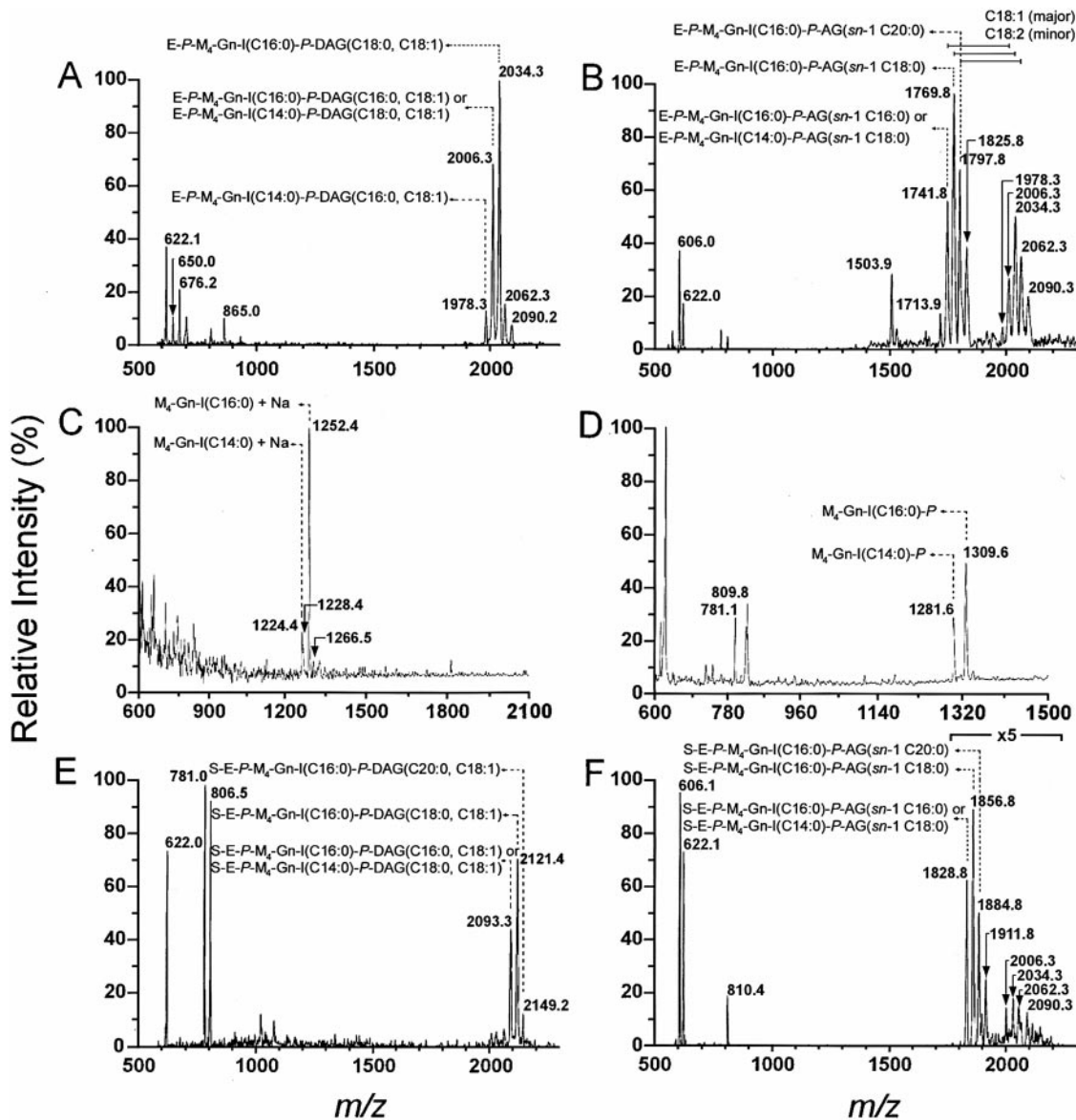
Figure 2. Mass spectrometry analysis of *P. falciparum* GPIs. HPLC- and HPTLC–purified GPIs were analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry in negative or positive ion mode. (A) Negative ion mass spectrum of the purified free GPIs (Fig. 1 B, lane 2). (B) Negative ion mass spectrum of phospholipase A_2–treated free GPIs showing (M-H)$^-$ ions of unconverted GPIs and GPIs lacking substituent at *sn*-2 position. (C) Positive ion mass spectrum of inositol-acylated glycan moiety released by HF treatment of GPIs. (D) Negative ion mass spectrum of inositol-acylated glycan moiety released by HF treatment of GPIs. (E) Negative ion mode mass spectrum of the amino acid–linked GPI fraction I (Fig. 1 B, lane 4). The mass spectrum of fraction II (Fig. 1 B, lane 4) contained (M-H)$^-$ ions at *m/z* 2147.3 at significantly higher proportions compared with fraction I, in addition to an ion at *m/z* 2175.3 (not shown). (F) Negative ion mass spectrum of phospholipase A_2–treated amino acid–linked GPIs showing (M-H)$^-$ ions of unconverted GPIs and GPIs lacking substituent at *sn*-2 position. (A–F) The major GPI(s) that represent(s) the molecular ions are indicated. E, ethanolamine; P, phosphate; M_4, Man_4; Gn, GlcN; I, inositol; AG, acylglycerol; DAG, diacylglycerol; S, serine. (G) The cleavage sites for the attachment of GPIs in *P. falciparum* MSPs.

1,228.7 and 1,266.8 represent (M+H$^+$) and (M+K$^+$) ions of the carbohydrate with palmitoyl substitution. Of several mass spectra recorded, some (not shown) gave a reasonable signal at *m/z* 1,200.4, which was assigned to the (M+H$^+$) ion of the carbohydrate with myristoyl substitution. A significant portion of the HF-released glycan moieties retained a phosphate ester group as indicated by the negative ion mode spectrum, which showed molecular ions at *m/z* 1,280.0 and 1,308.1 (Fig. 2 D). The phosphate ester group that survived HF treatment is likely the one linked to inositol because the adjacent protonated GlcN residue will render the hydrolysis of the phosphate ester bond to be kinetically slower. These results established that the inositol residue is substituted with palmitate (~90%) and myristate

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($\sim$10%). The diacylglycerol moiety released on treatment of GPI with HF contained predominantly C18:0 and C18:1 (two isomers), minor proportions of C14:0, C16:0, C18:2, C20:0, and C22:0, and unidentified acids. As C18:1 and C18:2 are present at *sn*-2 (see above), the remainders of the above acids should be located at *sn*-1.

The mass spectra of the HPTLC-purified, amino acid–linked GPIs (fraction I in Fig. 1 B, lane 4) containing one amino acid after pronase digestion showed prominent molecular ions that are 87 mass units higher than those of free GPIs (compare Fig. 2, E and F, with A), indicating that these GPIs contain a Ser residue. GPIs with attached Asn were also evident from $(M-H)^-$ ions at m/z 2120.3, 2148.3, and 2176.3 in the mass spectrum (not shown) of fraction II (Fig. 1 B, lane 4). This agrees with the predicted amino acids in GPI-anchored surface proteins (13–15), MSP-1, MSP-2, and MSP-4 (Fig. 2 G). Phospholipase A_2 treatment caused reduction by 264.5 mass units in the m/z of each of the molecular ions (Fig. 2 F), confirming the presence of predominantly C18:1 acyl residues at *sn*-2.

The acyl substituents in the GPIs were also studied by metabolic labeling with [^{3}H]myristic, [^{3}H]palmitic, [^{3}H]palmitoleic, [^{3}H]stearic, [^{3}H]oleic, [^{3}H]linoleic, and [^{3}H]arachidonic acids. All except arachidonate were incorporated into the parasite GPIs (Fig. 3 and data not shown). Treatment of the GPIs labeled with [^{3}H]myristic and [^{3}H]palmitic acids with phospholipase A_2 showed the presence of incorporated label only in the *lyso*-GPIs; the released fatty acids were not radioactive (Fig. 3). Upon treatment with HF, the radioactivity was found to be present on both the diacylglycerol and inositol carbohydrate moieties (not shown). In the case of [^{3}H]stearic acid, the radioactivity was incorporated only to the diacylglycerol moiety (not shown). These results agree with the presence of saturated fatty acids at *sn*-1 and at C-2 of inositol, but not at *sn*-2, established by mass spectrometry. The data also suggest that the parasite does not incorporate saturated fatty acids as unsaturated acids to the GPIs they synthesize or vice versa.
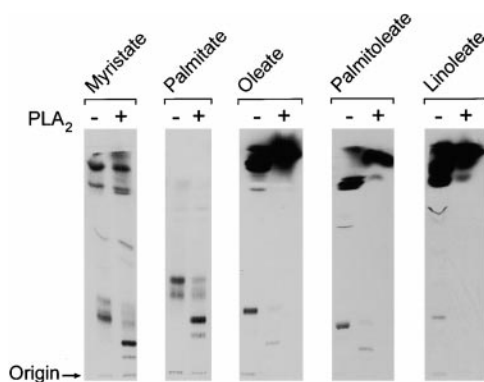
Treatment of [^{3}H]palmitoleic, [^{3}H]oleic, and [^{3}H]linoleic acid–labeled GPIs with phospholipase A_2 released >90% of the incorporated activity as free acid; the *lyso*-GPIs contained negligible radioactivity (Fig. 3). Treatment with HF showed that most of the incorporated radioactivity was on the diacylglycerol moiety, and the inositol carbohydrate moiety was not radioactive (not shown). These results agree with the presence of unsaturated fatty acyl residues at *sn*-2 as indicated by mass spectrometry of phospholipase A_2–treated GPIs. The incorporation of [^{3}H]palmitoleic acid to *sn*-2 is most likely after its conversion to cis-vaccenic acid; this agrees with the presence of cis-vaccenic acid at *sn*-2 of the GPIs (see above) and the ability of the parasite to synthesize this acid (42).

Based on the above data, the structure shown in Fig. 4 is proposed for *P. falciparum* GPIs.

TNF-α–inducing Activity of P. falciparum GPIs. Previous studies have shown that *P. falciparum* GPIs can induce the expression of proinflammatory cytokines in macrophages (27–29). To determine if highly purified GPIs can induce biologic activity, cultured murine macrophages were incubated with 0.03–0.5 μM purified GPIs, and the amount of TNF-α in the culture medium was measured. Consistent with a previous finding (26), the purified parasite GPIs induced TNF-α (30–450 pg/ml) in a dose-dependent manner (not shown).

Analysis for the Natural Anti-GPI Antibody in Humans. To examine whether people living in malaria endemic areas express naturally elicited anti-GPI antibodies, we analyzed sera from 300 adults in Western Kenya and 50 USA adults not exposed to malaria. In ELISAs performed with a coating of 2 ng/well of HPLC-purified, free GPIs, all Kenyan adult sera exhibited moderate to high levels of GPI-specific antibodies, predominantly IgGs, whereas all USA adults completely lacked anti-GPI antibodies (Fig. 5). The anti-GPI antibody activity of Kenyan sera was dose dependent and saturable (Fig. 5). There was no significant difference in levels of GPI-specific antibody reactivity between
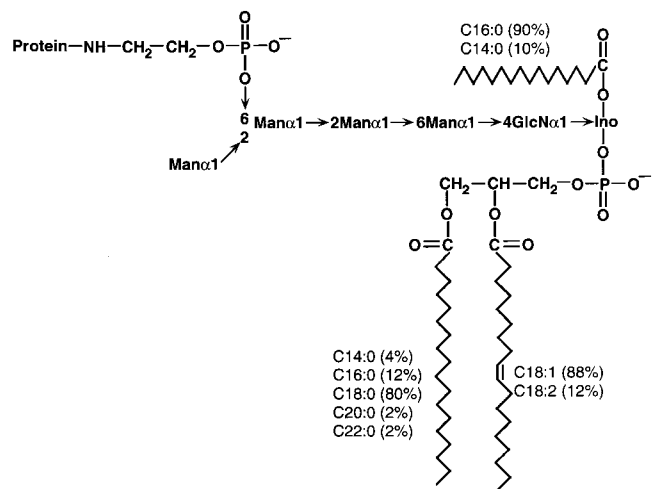

Figure 3. *P. falciparum* GPIs contain predominantly C18:1 fatty acid at *sn*-2. The parasites were metabolically labeled with various ^{3}H–fatty acids. Free GPIs were isolated, treated with bee venom phospholipase A_2 (PLA$_2$), and analyzed by HPTLC. −, untreated GPIs; +, phospholipase A_2–treated GPIs.

Figure 4. The proposed structures of *P. falciparum* GPIs. The fatty acids and their molar proportions are indicated.
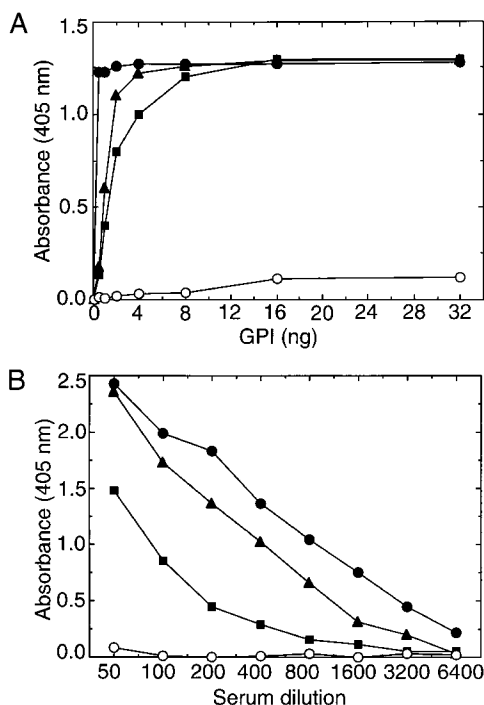
Figure 5. ELISA for naturally elicited anti-GPI antibodies in human sera. The HPLC-purified, free GPIs were coated onto 96-well microtiter plates either at the indicated amounts per well (A) or at 2 ng per well (B). The wells were blocked with TBS-casein, and then incubated with human sera, 1:100 or serially diluted with TBS-casein, 0.05% Tween 20. After washing the plates, the bound antibodies were detected with HRP-conjugated goat anti–human IgG (H and L chains) using ABTS substrate. ●, Kenyan adult sera 1; ▲, Kenyan adult sera 2; ■, Kenyan adult sera 3; ○, USA adult control serum.

Figure 6. Inhibition of anti-GPI antibody binding to GPIs by phospholipids. The HPLC-purified, free GPIs were coated on to microtiter plates (2 ng), blocked with TBS-casein, and overlaid with representative Kenyan sera (1:200 diluted) incubated with the indicated phospholipids and purified GPIs. The bound antibodies were measured by HRP-conjugated goat anti–human IgG (H and L chains) using ABTS substrate. Shown are the data from a representative of 10 different sera analyzed. Black bar, without inhibitor (Control); hatched bars, 2.5 ng; white bars, 5 ng; gray bars, 10 ng; stippled bars, 20 ng.

HPLC-purified free and amino acid–linked GPIs, indicating that the immunoreactivity is not due to contamination by parasite proteins. More than 85% of Kenyan adult sera also contained lower but significant levels of GPI-specific IgM antibodies (Naik, R.S., unpublished results). The erythrocyte membrane debris, saponin lysate, and ghosts were nonreactive to the infected Kenyan sera (data not shown).

When ELISA was performed using several commercially available phospholipids, PIs from bovine liver and soybean that lack acyl substituent on inositol, PGs, and CL, Kenyan adult sera showed a low level of activity: ∼5–15% of that observed with *P. falciparum* GPIs. At all coating concentrations tested (2–50 ng/well), these compounds showed similar low levels of antibody reactivity. Prior incubation of Kenyan sera with 2.5–20 ng/ml of PIs, PGs, or CL, and then ELISA using plates coated with HPLC-purified free GPIs showed in all cases ∼5–15% lower antibody binding activity, irrespective of the concentrations of lipid used (Fig. 6). However, prior incubation with the parasite GPIs inhibited binding by up to 75% in a dose-dependent manner (Fig. 6). Together, these results suggest that Kenyan adult sera have low levels of reactivity to common acylated phosphoglycerols. This is not surpris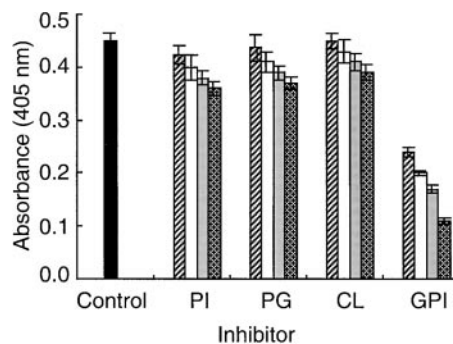ing because of the polyclonal nature of GPI-specific antibodies, which are expected to contain antibodies to the phosphoglycerol portion of GPIs that are common to this class of molecules.

TLC immunoblotting of the HPTLC-purified GPIs confirmed the specificity of the seroreactivity: adult Kenyan sera reacted with the GPIs, whereas control sera from USA adults were nonreactive (Fig. 7). Furthermore, the Kenyan adult sera were nonreactive to bovine liver and soybean PIs that lack an acyl substituent on inositol, PGs, and CL (Fig. 7, and data not shown); the apparent lack of reactivity to these lipids on TLC plates could be due to a low level of sensitivity. Furthermore, treatment of the GPIs with HNO_2 shifted the immunoreactivity to the position of the PI moiety, suggesting that this portion of the molecule is antigenic (Fig. 7).

The identity of the antigenic part of GPIs was further confirmed by inhibition of seroreactivity to intact GPIs using carbohydrate and lipid moieties of GPIs isolated after HNO_2 fragmentation. The carbohydrate moiety inhibited antibody binding by only ∼5% at a 10-fold higher concentration compared with that of the coated, intact GPI. The lipid moiety, at the same coating concentration as that of intact GPIs, showed >70% antibody-binding activity, indicating that the PI moiety is the antigenic structure. These results suggest that acylated inositol is the major moiety that recognizes the naturally elicited anti-GPI IgGs.

Anti-GPI Antibody Response and Acquired Resistance to Malaria Pathogenesis. To determine whether the susceptibility of young children in malaria endemic areas is related to the absence of GPI-specific antibodies, sera taken every month after birth for 4 yr from a cohort of 48 children were analyzed (8). The results were compared with those of sera from 100 siblings (7–8 yr) and 50 nonpregnant mothers (20–25 yr) (Fig. 8 A). Malaria parasitemia and clinical parameters (hemoglobin and fever) were followed every 2 wk in this community-based cohort. Whereas a case-control study design compares extremes, this nonbiased design de-
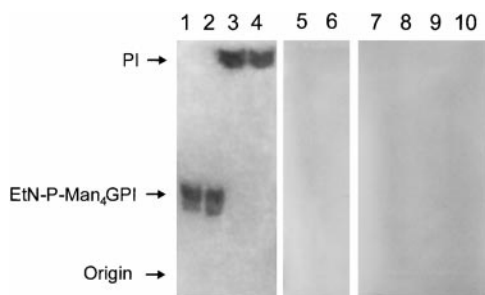
Figure 7. HPTLC immunochromatogram of *P. falciparum* GPIs. The HPTLC-purified, free GPIs (100 ng each) were chromatographed on HPTLC plates, blocked with 1% BSA, and incubated for 2 h in 1:100-diluted sera. The bound antibodies were detected with ^{125}I-labeled goat anti–human IgG (5 μCi/ml). Lanes 1 and 2, free and amino acid–linked GPIs, respectively, treated with Kenyan adult sera; lanes 3 and 4, HNO$_2$-released lipid from free and amino acid–linked GPIs, respectively, treated with Kenyan adult sera; lanes 5 and 6, free and amino acid–linked GPIs, respectively, treated with control USA adult sera; lanes 7 and 8, PIs from bovine liver and soybean, respectively; lane 9, PG; lane 10, CL, treated with Kenyan adult sera. Shown are representatives of 10 Kenyan and USA adult sera analyzed. Parasite lipids other than GPIs and their intermediates, extracted with chloroform/methanol (2:1, vol/vol), and glycolipids from control erythrocytes were completely nonreactive to Kenyan sera (not shown).

Figure 8. Age-dependent anti-GPI antibody response in people living in malaria endemic area. Sera from a cohort of children and adults were analyzed by ELISA using HPLC-purified, free GPIs (see Fig. 5). An antibody level (OD) greater than the mean of USA adult control sera plus 2 SD was considered positive. (A) Percentage of individuals in the following anti-GPI antibody responder categories: negative at both 1–3-mo-spaced time points sampled, negative (white bars); positive at one time point, intermittent (hatched bars); and positive at both time points, positive (black bars). $n \geq 40$ in each age group between ~0.5 and 3.5 yr, and $n = 100$ and 50 in the 7–8-yr and 20–25-yr age groups, respectively. A χ^2 test found that the antibody responder category was different among age groups ($P < 0.001$). (B) Mean hemoglobin (g/dl, white bars), temperature ($-29.5°$C, hatched bars), and anti-GPI antibody level [$\log_{10}$(OD+1)] (●) for the indicated age groups. Analysis of variance found that the anti-GPI antibody level, hemoglobin, and temperature were different among age groups ($P < 0.0001$).

termined if an association between anti-GPI antibodies and malaria pathogenesis was detectable at a population level. We carefully controlled for *P. falciparum* transmission, antimalaria drug treatment, and age. By sampling the same child over 4 yr, we found that, within an individual, anti-GPI antibody responses correlate with protection against malaria-attributable hemoglobin loss and febrile illness (see below).

We performed ELISA on sera from each child on all monthly samples and for siblings and adults at two time points spaced 1–3 mo apart to determine the anti-GPI antibody level and the persistence of the antibody response. For comparison and correlation analysis, results of four time points (0.5, 1.5, 2.5, and 3.5 yr old), based on the sampling scheme employed for the 7–8-yr-olds and the mothers, were used for children (Fig. 8 A). The data show that ~50% of the children under 2 yr lacked anti-GPI antibodies (negative responders, defined in the legend to Fig. 8), ~40% had short-lived antibody responses (lasted in the circulation for only 1–3 mo, intermittent responders), and only ~10% had persistent (long-lived) antibody responses (positive responders) (Fig. 8 A). In contrast, ~75% of the 7–8-yr-old children and all adults exhibited a persistent anti-GPI antibody response. The level of anti-GPI IgG increased with increasing age from infancy to adulthood, whereas mean hemoglobin density increased and fever (temperature) decreased with age up to 7–8 yr of life (Fig. 8 B). Thus, the presence of long-lived, high levels of anti-GPI antibodies in sera parallels the naturally acquired resistance against malaria pathogenesis.

We found that anti-GPI antibody response, age, and parasite density were independently associated with hemoglobin ($P < 0.0147$). Age influenced the anti-GPI antibody responder category; however, it was not significant ($P <$
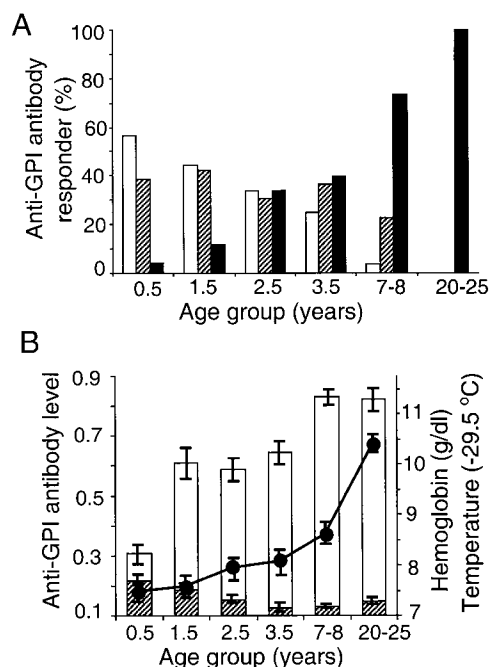
0.0581), suggesting that the association between the antibody responder category (as defined in the legend to Fig. 8) and hemoglobin was not just a reflection of age. For example, using a general linear model, it was estimated that 6-mo-old children in the positive antibody responder category had 2.37 g/dl of hemoglobin more than 6-mo-olds in the intermittent responder category. Antibody responder category and parasite density were independently associated with temperature ($P < 0.0012$). 6-mo-olds in the negative antibody responder category had an estimated temperature 0.71°C higher than the individuals in the intermittent antibody responder category. Fig. 9 shows the associations between anti-GPI antibody responder category and malaria-attributable pathology in children 0.5–3.5 yr old. In each parasitemia category, febrile illness increased and hemoglobin level decreased in children without antibodies or with only short-lived antibodies; however, in children with persistent antibodies, febrile illness was lower and hemoglobin level was higher. These results strongly support the hypothesis that circulating anti-GPI antibodies neutralize the toxic effects of parasite GPIs.
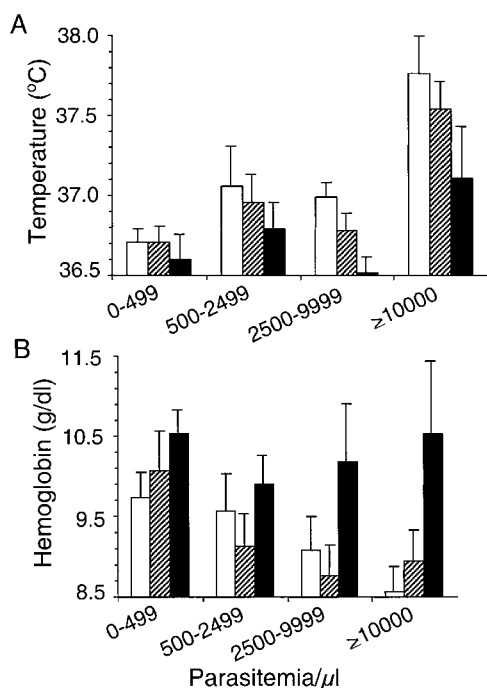
Figure 9. Serum anti-GPI antibody response and resistance to malaria in children 0.5–3.5 yr of age. A general linear model (GLM) was used to investigate the correlation between anti-GPI antibody responder category and temperature or hemoglobin level, while controlling for age and parasite density. (A) Mean temperature (°C) for 0.5–3.5-yr-old negative (white bars), intermittent (hatched bars), and positive (black bars) anti-GPI antibody responder categories. (B) Mean hemoglobin (g/dl) for 0.5–3.5-yr-old negative (white bars), intermittent (hatched bars), and positive (black bars) anti-GPI antibody responder categories (see legend to Fig. 8). The anti-GPI antibody responder category and parasite density were independently associated with hemoglobin ($P < 0.0147$ and $P = 0.0000$, respectively). The associations were independent of the nonsignificant association between age and hemoglobin ($P = 0.0581$). Antibody responder category and parasite density were independently associated with temperature ($P = 0.0012$ and 0.0001, respectively). Age was not found significantly correlated with temperature ($P > 0.0581$).

Discussion

This paper reports two major findings. (a) The results presented here establish for the first time that individuals residing in malaria-endemic areas develop a *P. falciparum*–specific anti-GPI antibody response, whereas people not exposed to the malaria parasite do not have these antibodies; in addition, anti-GPI antibody responses are correlated with protection against malaria–related febrile illness and hemoglobin loss. This agrees with the previous reports describing *P. falciparum* GPIs as pathogenicity factors based on their ability to induce a spectrum of proinflammatory cytokine responses and cause malaria symptoms. (b) Another important finding is elucidation of complete structures of the parasite GPIs by direct biochemical analysis and mass spectrometry. Thus, our results identified two novel structural features of *P. falciparum* GPIs: the presence of palmitate and myristate on C-2 of inositol, and the presence of predominantly oleic and minor amounts of cis-vaccenic acid and C18:2 at *sn*-2.

The purification of *P. falciparum* GPIs to homogeneity was crucial for structural determination as well as to obtain unambiguous evidence for biologic activity and establish the presence and specificity of naturally elicited antibodies in the sera of people living in malaria endemic areas. The key steps employed for isolation and purification of GPIs were: (a) growing of mycoplasma-free cultures to high levels of parasitemia and enrichment of infected erythrocytes; (b) metabolic labeling of GPIs and the use of human sera containing anti-GPI antibodies to follow purification steps; (c) use of sterile water and buffers and high quality organic solvents to exclude external contamination; and (d) siliconizing glassware to avoid loss due to surface adsorption. The mass spectrometry results presented here show that the purified GPIs are homogeneous.

We determined the structures of *P. falciparum* GPIs by subjecting the [^{3}H]GlcN- and ^{3}H–fatty acid-labeled GPIs to various standard degradative procedures, including their susceptibility to nitrous acid, HF, alkali, jack bean α-mannosidase, and GPI-specific phospholipase D, by determining fatty acid composition and finally by direct mass spectrometry. The results enabled us to propose the structures shown in Fig. 4 for the parasite GPIs. The structure of the core glycan is the same as determined previously (19, 20). However, the acyl substituents are different from those reported previously, based on radiolabeling, with palmitate at both *sn*-1 and *sn*-2 and predominantly myristate on inositol (21). As shown in Fig. 4, palmitate is the major acyl substituent with minor proportions of myristate on inositol of the parasite GPIs; GPIs with acylated inositol residues from other sources reported to date contain only palmitate on the inositol residue (12). The parasite GPIs contain unsaturated acyl substituents at *sn*-2 (major C18:1 and minor C18:2) and predominantly C18:0 and a range of variable size saturated acyl residues at *sn*-1. With respect to the nature of the acyl residue at *sn*-2, the parasite GPIs resemble the GPI of *T. cruzi* trypomastigote mucin that has a potent cytokine-inducing property (30). Another unusual feature of the parasite GPIs is the presence of cis-vaccenic acid at *sn*-2.

The parasite GPIs differ significantly from those of humans with respect to both the acyl substituents and the carbohydrate moiety (12). The GPI moieties of human erythrocyte proteins (the erythrocytes do not contain detectable levels of free GPIs), CD59, and acetylcholine esterase contain exclusively a C18 alkyl substituent at *sn*-1, C22:4 at *sn*-2, and palmitate on inositol; the carbohydrate moieties contain one or two extra phosphoethanolamine as well as βGalNAc residues (12). The GPI of human spleen CD52 contains a diacylglycerol moiety and lacks GalNAc; however, this differs from the parasite GPIs with respect to the type of fatty acid at *sn*-2 (C22:4, C22:5, and C22:6) and contains phosphoethanolamine on the first mannose residue (12). These structural differences may contribute to the observed naturally elicited immunologic responses against the parasite GPIs in humans.

Although Schofield et al. have shown that *P. falciparum* GPIs can transduce signals to elicit inflammatory cytokine

responses (27–29), there have been concerns as to whether the observed activity was due to contamination (parasite, erythrocytes, and/or mycoplasma origin [43]). These concerns were based on the observations by some investigators that aqueous buffer extracts of parasite cultures, presumed to have extracted the parasite GPIs upon boiling, could not elicit TNF-α. Thus, they have argued that the cytokine-inducing property of *P. falciparum* was due to unknown components. However, it should be noted that GPIs can only be extracted with organic solvents. Because of this existing controversy, we tested TNF-α induction by the highly purified parasite GPIs. The results presented in this paper clearly show that the purified *P. falciparum* GPIs can induce TNF-α in macrophages. This activity is consistent with the recent finding by Almeida et al. that a highly purified GPI moiety of *T. cruzi* trypomastigote mucin induces TNF-α (30) and confirms the previous finding by Schofield et al. (26). Furthermore, studies by Almeida et al. also show that C18:1 and/or C18:2 acyl substituent at *sn*-2 in the *T. cruzi* GPIs is critical for TNF-α–inducing activity (30). As the *P. falciparum* GPI also contains C18:1 (major) and C18:2 (minor) at *sn*-2, it is possible that these acyl substituents contribute to the toxic property of the parasite GPIs.

In malaria endemic areas, younger children have the highest risk of developing severe malaria, whereas older children and adults rarely develop severe disease despite repeated exposure and significant parasitemia (23). People in nonmalarious regions completely lack this resistance, suggesting that the protection is due to a parasite-specific response acquired through repeated infections. In the Western Kenyan population studied here, we found that adults have *P. falciparum* parasitemia >14% of the time, whereas children <4 yr old have parasitemia >60% of the time. The density of parasitemia is lower in adults and, importantly, the level of parasitemia that can be tolerated without causing febrile illness or anemia is higher in adults (Branch, O.H., unpublished). As *P. falciparum* GPIs are pathogenicity factors (26–29), the resistance of adults to malaria illness may be related to a GPI-specific protective immunity.

All the Kenyan adult sera analyzed contained high levels of GPI-specific IgGs, whereas all 50 USA adult sera completely lacked such antibodies. The antibody response was highly specific to GPIs and their intermediates; other phospholipids of the parasite showed only low levels of immunoreactivity. Several phospholipids, including PIs, PGs, and CL, showed only 5–15% of the immunoreactivity exhibited by the parasite GPIs, which appears to be due to the polyclonal nature of the antibodies reacting with the common epitopes. Previously, several studies have reported the presence of significant levels of antibodies that bind phospholipids either directly or via binding of serum β2-glyco-protein I (44). In those studies, lipids were coated with several micrograms per well for ELISA. It is known that proteins and antibodies can nonspecifically bind to lipids when coated at high density. In contrast, our study used 0.5–2 ng/well of GPIs for ELISA and 100 ng for TLC immunoblots. Moreover, in previous studies, plates coated

with lipid antigens were incubated with sera diluted with buffers without detergent. Under such conditions, we found high levels of nonspecific activity. Thus, our study clearly demonstrates that the identified IgGs are specific to GPIs.

Our results also establish that the PI portion of the GPIs contributes significantly to immunogenicity. The removal of the *sn*-2 fatty acid from the GPIs did not affect antibody reactivity (Vijaykumar, M., unpublished results). As treatment with HF abolished immunoreactivity, it appears that the acylated inositol phosphate is the immunogenic portion of the molecule. This agrees with the previous finding that antibodies raised against PIs can inhibit the induction of TNF-α by *P. falciparum* extracts (45). These results are important in that if an *sn*-2 acyl substituent is indeed required for cytokine-inducing activity, then it may be possible to synthesize nontoxic molecules for therapeutic purposes.

The TNF-α–inducing activity of GPIs and the correlation between the general resistance of adults in endemic areas to malaria pathogenesis and the presence of serum anti-GPI antibody response suggest that the acquired immunity is related significantly to the anti-GPI antibodies. This prediction agrees with the lack of such an antibody response in the majority of children <4 yr old, the risk of children developing severe malaria, and the correlation between the gradual acquisition of the antibody response in an age-dependent manner (>80% of the 7–8-yr-olds having high levels of serum antibody) and protection against malaria with age. Thus, a direct correlation between anti-GPI antibodies and malaria-related pathology could be observed in young children.

High levels of antibodies against several other antigens such as MSP-1, EBA-175, and circumsporozoite protein are also present in adults, and therefore it may be argued that the anti-GPI antibody response is not independent from those antibodies. However, it is important to consider that the anti-GPI antibody response is related to antidisease immunity, whereas antibodies against parasite proteins studied to date are involved in antiparasite immunity (controlling parasite burden). Furthermore, whereas ~80% of children <2 yr of age either lack or contain very low levels of short-lived anti-GPI antibodies, these children can have high levels of antibodies against MSP-1 and other proteins (8, 9; and Branch, O.H., unpublished results). Although there was a correlation between the levels of MSP-1 and EBA-175 antibodies and protection against parasite density, these antibody responses were not related to anti-GPI antibody response, protection against febrile illness, and hemoglobin loss at any given parasite density.

Almost all malaria vaccine development efforts currently being pursued use parasite proteins in a multicomponent formulation, aimed at providing immunity against infection (antiparasitic [5]). On the other hand, by blocking the toxic effects of the parasite GPIs, a GPI-based vaccine might significantly reduce malaria pathogenesis. The identification of the PI moiety as the functional part of the molecules should significantly simplify any approach towards the development of antidisease measures.

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