A prospective analysis of the Ab response to *Plasmodium falciparum* before and after a malaria season by protein microarray

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T he parasite, *Plasmodium falciparum* (*Pf*) is responsible for over 500 million malaria episodes annually, approximately 1 million of which result in death, the majority among African children (1). Those who survive malaria early in life eventually acquire resistance to the disease after repeated exposure to the parasite (2), but the mechanisms that underlie this immunity remain poorly understood (3). Early studies showed that purified IgG from malaria-immune adults, when transferred to children acutely ill with malaria, reduced fever and parasitemia (4), thus indicating that Abs against *Pf* proteins play a critical role in controlling the blood stage of the infection. However, which of the 5,400 possible *Pf* proteins (5) elicit the production of protective Abs is unclear.

To study Ab responses to human pathogens on a genome-wide scale, our laboratory (P.L.F.) developed a high-throughput cloning and expression system that permits the construction of protein microarrays representing a variety of important viruses, bacteria, and parasites, including vaccinia virus (6, 7), *Borrelia burgdorferi* (8), *Burkholderia pseudomallei* (9), and *Pf* (10). In a single assay, the arrays can be probed with serum from infected or vaccinated individuals to identify detailed Ab profiles associated with infection or protection from disease. These studies demonstrated that protein microarrays are a rapid and accurate method of identifying vaccine (7, 10) and serodiagnostic (8, 9) targets, defining immunogenicity of vaccine formulations, and identifying correlates of protection and surrogate end points in animal models and humans.

A number of studies have been carried out in areas endemic for malaria to identify a correlation between malaria immunity and an individual’s *Pf*-specific Abs. These studies have been constrained to measuring Abs to the relatively few *Pf* proteins made available through traditional cloning methods (<0.5% of the proteome) (11). Thus far, correlations between Abs to the relatively small number of *Pf* proteins that have been tested and malaria immunity have not been firmly established, suggesting that Abs against these proteins do not play a role in protective immunity or, more likely, that Abs against single-parasite proteins are insufficient to confer protection.

To address these knowledge gaps, we used *Pf* genome sequence data (5) to construct a protein microarray representing ~23% of the *Pf* proteome (1,204 known and hypothetical proteins). In a prospective study in Mali of 220 individuals aged 2–10 years and 18–25 years in Mali before and after the 6-month malaria season, episodes of malaria were detected by passive surveillance over the 8-month study period. Ab reactivity to *Pf* proteins rose dramatically in children during the malaria season; however, most of this response appeared to be short-lived based on cross-sectional analysis before the malaria season, which revealed only modest incremental increases in Ab reactivity with age. Ab reactivities to 49 *Pf* proteins measured before the malaria season were significantly higher in 8–10-year-old children who were infected with *Pf* during the malaria season but did not experience malaria (n = 12) vs. those who experienced malaria (n = 29). This analysis also provided insight into patterns of Ab reactivity against *Pf* proteins based on the life cycle stage at which proteins are expressed, subcellular location, and other proteomic features. This approach, if validated in larger studies and in other epidemiological settings, could prove to be a useful strategy for better understanding fundamental properties of the human immune response to *Pf* and for identifying previously undescribed vaccine targets.

Results

Study Site and Clinical Outcomes. This study was conducted in the rural village of Kambia, Mali. Details of the study site have been reported elsewhere (12). The site was selected because of the sharp demarcation between the 6-month malaria season and the

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6-month dry season, during which there is either intense or little to no Pf transmission, respectively (13), thus allowing for comparisons of immunological parameters in the presence or absence of Pf exposure. In May 2006, just before the malaria season, 225 individuals between the ages of 2–10 years and 18–25 years were enrolled in the cohort study after random selection from an age-stratified census of the entire village population of 1,500. Over the subsequent 8 months, study participants were instructed to report symptoms of malaria at the village health center, staffed 24 h/day by a study physician. Of the 495 unscheduled clinic visits, 298 were classified as malaria cases for the purpose of study analysis, as defined by an axillary temperature ≥37.5 °C, Pf asexual parasitemia ≥5,000 parasites per microliter, and a nonfocal examination by the study physician. Only 5 of the 298 malaria episodes met the World Health Organization (WHO) criteria for severe malaria (14). All individuals presenting with a blood smear positive for Pf and signs or symptoms of malaria were treated according to international standards, regardless of the parasitemia level. As expected, malaria risk during the Pf transmission season decreased with age (Fig. 1). The primary end point for analysis was whether or not an individual experienced malaria during the 8-month study period. The percentage of individuals experiencing ≥1 malaria episodes was 86.3% in 2–4 year olds, 86.5% in 5–7 year olds, 60.8% in 8–10 year olds, and 8.2% in 18–25 year olds (Fig. 1). Several features of this study favored unbiased detection of malaria episodes: (i) the study population was an age-stratified random sample representing 15% of all individuals living in a small (1 km²), well-circumscribed, nonmigratory community; (ii) follow-up at scheduled visits was >99% for children and 82% for adults, indicating a high degree of study awareness and participation; (iii) antimalarial drugs were provided exclusively by the study investigators, who were available at all times at the only easily accessible health care facility; and (iv) the average distance from individuals’ homes to the study clinic was 0.38 km (range: 0.13–0.88 km), minimizing logistic barriers to study participation.

**Analysis of Broad Pf-Specific Ab Profiles Before and After the Malaria Season.** To assess the validity of Ab reactivity against proteins expressed in the high-throughput translation system, we included three well-characterized correctly folded Pf proteins being developed as malaria vaccine candidates on the same microarray chip. We found that Ab reactivity against these malaria vaccine candidates correlated with Ab reactivity against the same proteins expressed in the high-throughput system (SI Text and Fig. S1). The microarray assay was highly reproducible from chip to chip (SI Text and Fig. S1). Each microarray chip contained positive and negative controls and an IgG standard curve to normalize data from arrays probed at different times (SI Text and Fig. S2).

We examined Ab reactivity to the 2,320 Pf proteins (representing 1,204 unique proteins) on the microarray in plasma collected before and after the 6-month malaria season. Of the 225 individuals enrolled in the study, microarray data were available from 220 individuals before the malaria season and 194 individuals after the malaria season. The level of Ab reactivity to 491 of the 2,320 proteins measured before the malaria season exceeded 2 SDs above the negative control (a rapid translation reaction containing an empty plasmid vector). These 491 proteins are listed in Table S1 and referred to here as immunogenic proteins. Based on mass spectrometry data (15) obtained from PlasmoDB (www.plasmodb.org), the life cycle stage at which these 491 proteins are maximally expressed is as follows: sporozoite (25.2%), merozoite (5.5%), trophozoite (16.8%), gametocyte (20.6%), and unknown (31.9%) (Fig. S3). Gene ontological analysis indicated that ~40% of the immunogenic proteins are expressed in the membranes or host erythrocyte (Fig. S3) and that they are over-represented in the biological process categories of “pathogenesis,” “cytoadherence to microvasculature,” “antigenic variation,” and “rosetting” (Fig. S3 and Table S2). In a study that used a smaller version of the same array platform (250 proteins), Doulan et al. (10) identified 32 “serodominant” proteins in the serum of Pf-exposed Kenyan adults, 26 of which were identified as immunogenic in this study (overlapping proteins are indicated in Table S1).

As is apparent from the heat maps of arrays probed with plasma collected before and after the malaria season, both the breadth and intensity of Ab reactivity to the 491 immunogenic proteins increased with age (Fig. 2A) and from before to after the malaria season (Fig. 2B). The average number of proteins recognized by Pf-exposed individuals increased with age, both before (P < 0.0001; Fig. 2C) and after (P < 0.0001; Fig. 2C) the malaria season. The average number of proteins recognized by children in each age group increased from before to after the malaria season (P < 0.0001 for each comparison; Fig. 2C), whereas the number recognized by adults did not increase significantly (Fig. 2C). Similarly, the number of proteins recognized by at least 90% of individuals in each age group increased with age and Pf transmission (Fig. 2D). By the end of the malaria season, nearly all the 491 immunogenic proteins were recognized by at least 50% of individuals 8 years of age and older (Fig. S4). Pf-naïve adults (n = 27) recognized an average of 203 of the 491 immunogenic proteins (Fig. 2C); however, as shown below, the level of Ab reactivity against these proteins was relatively low. Moreover, only 24 (4.9%) of the 491 immunogenic proteins were recognized by 90% of Pf-naïve adults (Fig. 2D), in contrast to the 346 proteins (70.5%) recognized by 90% of Pf-exposed adults after the malaria season (Fig. 2D). Of note, Ab reactivity to the 24 proteins recognized by 90% of Pf-naïve adults increased, along with Ab reactivity to all 491 immunogenic proteins in Pf-exposed individuals (Fig. S4), suggesting that the low level of Ab reactivity in samples from Pf-naïve adults may be attributable, in part, to cross-reactivity of Abs generated in response to other protozoa such as Toxoplasma gondii (16), a relatively common infection in the United States (17), rather than to nonspecific binding.

We also quantified the level of Ab reactivity to the 491 immunogenic proteins with age and in response to Pf transmission. For each study participant, we calculated the average Ab reactivity to the 491 immunogenic proteins before and after the malaria season and then plotted the mean of these values within each 1-year age group (Fig. 2E). The mean level of Abs increased during the malaria season in each age category, but the increase was only statistically
significant ($P < 0.05$) for children aged 2–8 years. The Ab reactivity level of $Pf$-naive adults was relatively low, similar to that of children aged 2–4 years before the malaria season (Fig. 2E), but lower than that of individuals aged 5 years and older before the malaria season ($P < 0.01$ for each comparison) and lower than that of all children and adults after the malaria season ($P < 0.01$ for each comparison).

In comparing the levels of Abs before the malaria season in children aged 2 and 3 years, most of the increase in Ab levels from before to after the malaria season appeared to be short-lived, decreasing over the 6-month dry season during which there is little to no $Pf$ transmission. However, the level of $Pf$-specific Abs before the malaria season was slightly higher in children aged 3 years vs. 2 years, suggesting that a small portion of the Abs acquired by 2-year-old children over the malaria season persisted for 6 months in the absence of $Pf$ exposure. This general pattern of rapid seasonal rise in Ab levels and gradual acquisition of long-lived Abs at the end of the dry season continued to adulthood; at that point, Ab reactivity was substantial at the end of the dry season. Purified Epstein–Barr nuclear antigen-1 (EBNA-1) was printed on the same array. Abs specific for EBNA-1 were detected in subjects of all ages, but consistent changes in Ab reactivity from before to after the malaria season were not observed (Fig. S5). Importantly, in children aged 2–10 years, the mean combined Ab reactivity to the 491 immunogenic $Pf$ proteins (reactivity to the negative control subtracted) measured before the malaria season increased with age. In both children and adults, the level of Ab reactivity increased from before to after the malaria season. Statistically significant ($P < 0.05$) increases are indicated by an asterisk.

**Fig. 2.** Impact of age and $Pf$ transmission on $Pf$-specific Ab profiles. Heat maps of proteins analyzed for immunoreactivity across plasma samples collected before (A) and after (B) the malaria season show that the breadth and intensity of Ab reactivity increases with age and in response to $Pf$ transmission. The 491 immunogenic proteins are represented in rows in descending order of immunoreactivity. Individual plasma samples are in columns and grouped by age in years (2–4 yrs, 5–7 yrs, 8–10 yrs, and 18–25 yrs). Within each age group, samples are sorted by increasing average immunoreactivity. Red indicates positive immunoreactivity, black indicates intermediate immunoreactivity, and green indicates no immunoreactivity. (C) Of the 491 immunogenic proteins, the average number recognized by $Pf$-exposed individuals increased with age both before ($P < 0.0001$) and after ($P < 0.0001$) the malaria season. Significant increases in the number of proteins recognized from before to after the malaria season within age groups are indicated by an asterisk ($P < 0.0001$ for all significant changes). (D) Number of proteins recognized by at least 90% of $Pf$-exposed individuals increased with age and $Pf$ transmission. (E) Average level of Ab reactivity to the 491 immunogenic $Pf$ proteins (reactivity to the negative control subtracted) measured before the malaria season increased with age. In both children and adults, the level of Ab reactivity increased from before to after the malaria season. Statistically significant ($P < 0.05$) increases are indicated by an asterisk.
Discussion

In this study, we used a protein microarray representing ~23% of the Pf proteome to gain insight into the kinetics of acquiring Pf-specific humoral immune responses and to identify Abs against known and hypothetical Pf proteins that may be associated with naturally acquired protection from uncomplicated malaria.

The approach described here addresses two technical bottlenecks that have hindered studies seeking to correlate naturally acquired malaria immunity with Ab responses to as many as 18 Pf antigens by ELISA (18, 19) or protein microarray (20), these studies evaluated already characterized malaria vaccine candidates.

We compared Ab profiles of children based on whether or not they had experienced malaria during the 8-month study period. We chose this end point, rather than the incidence of malaria or time to first malaria episode, because it represents the best possible clinical outcome for malaria vaccine candidates targeting the preerythrocytic and erythrocytic stages. Ab reactivity to 49 known and hypothetical Pf proteins was significantly higher in children aged 8–10 years who were infected with Pf during the study period but did not experience symptoms of malaria. Four of these proteins are being developed as vaccine candidates: sporozoite threonine-asparagine-rich protein (STARP), LSA-1, ring-infected erythrocyte surface antigen (RESA), and antigen 332. STARP is a conserved protein expressed primarily on sporozoites and, to a lesser extent, during the liver and early erythrocytic stages (21). STARP-specific IgG, whether acquired naturally or through irradiated sporozoite immunization, has been shown to inhibit sporozoite invasion of human hepatocytes (22). Although LSA-1 is expressed during the liver stage (23), where cell-mediated immunity likely predominates, LSA-1-specific IgG levels in individuals living in malaria endemic areas have been associated with protection against malaria (24). Furthermore, LSA-1-specific Ab responses have been detected in individuals vaccinated with irradiated sporozoites (25).

However, in the only phase I/II trial conducted to date, vaccination with recombinant LSA-1 did not protect against Pf experimental sporozoite challenge (26). RESA is released by the merozoite on erythrocyte invasion, at which time it interacts with the spectrin network of the host cell membrane (27). A blood-stage vaccine combining RESA, MSP-1, and MSP-2 showed some efficacy, reducing parasitemia in children enrolled in a phase I/IIb trial in Papua New Guinea (28), although the relative contribution of the three antigens to protection remains unclear. Antigen 332 is expressed in trophozoites and translocated to the erythrocyte membrane during the schizont stage (29). Abs against Pf332 have been shown to inhibit parasite grown in vitro (30) and have been associated with decreased parasitemia (31) and decreased malaria risk (32) in field studies.

We identified differential Ab reactivity against other conserved proteins that could be targeted as malaria vaccines. For example, that have evaluated this limited set of proteins have yet to establish a firm correlation between Ab responses and naturally acquired malaria immunity (11). Although more recent studies have sought to correlate malaria immunity with Ab responses to as many as 18 Pf antigens by ELISA (18, 19) or protein microarray (20), these studies evaluated already characterized malaria vaccine candidates.

Fig. 3. Pf-specific Ab profiles associated with protection from malaria. Heat map showing the difference in immunoreactivity measured before the malaria season against 49 proteins (in rows) in malaria-susceptible (Left, n = 29) and malaria-protected (Right, n = 12) children aged 8–10 years. (Right) Bar graph shows that the mean Ab reactivity against each of these proteins is higher in protected (red bars) vs. susceptible (blue bars) children (α = 0.05, false discovery rate-corrected). (Upper) For comparison, Ab reactivity against 5 proteins expressed in the rapid translation system that did not discriminate between protected and susceptible children is shown. These 5 proteins correspond to the malaria vaccine candidates CSP, LSA-3, MSP-1, MSP-2, and AMA-1. For the heat map, red indicates positive immunoreactivity, black indicates intermediate immunoreactivity, and green indicates no immunoreactivity. For the bar graph, data are mean ± SEM.
PFC0875w is predicted to be an ATP-binding cassette (ABC) transporter, and vaccination with ABC transporter proteins has been shown to protect against pathogenic bacteria in animal models (33, 34). PIESP2 (PFEO00060w) is a highly conserved protein of unknown function that is thought to be expressed on the surface of infected red blood cells (35). A protein with a MACPF-like domain (PFDE0330c) is an intriguing vaccine target because it plays a role in the traversal of hepatic sinusoids by sporozoites (36). Of the 25 var gene products included on the microarray, 2 were associated with protection: PF13_0003 and PF11_0008. Both belong to the group A var cluster, which is characterized by relative structural homogeneity (37), preferential expression in patients with severe malaria, and rapid induction of Abs (38). The analysis also identified 12 hypothetical proteins that were associated with protection, which highlights the potential value of this approach for identifying vaccine candidates. Further study of selected antigens identified by this strategy is needed to define their potential as malaria vaccine candidates. In addition, with the inherent bias in any single epidemiological study, larger studies in other settings are needed to validate this as an approach for identifying correlates of naturally acquired malaria immunity.

Ab reactivity to several intracellular proteins was significantly higher in the protected group. Abs to intracellular proteins are typically viewed as markers of past infection and not as evidence for vaccine potential, and it is possible that higher Ab reactivity against intracellular proteins among protected individuals is a marker of enhanced parasite killing. However, intracellular proteins of other parasites have been shown to induce protective Abs. For example, immunization with heavy chain myosin induced protection against Brugia malayi challenge in rodent models (39). Interestingly, in the present study, we found that Ab reactivity to Pf myosin was associated with protection from malaria. Thus, given how little is known about the infection biology of Pf in humans, it may be premature to dismiss immunogenic proteins categorically as potential vaccine targets based solely on subcellular location.

Ab reactivity to the malaria vaccine candidates CSP, LSA-3, AMA-1, MSP-1, and MSP-2 did not discriminate between protected and susceptible children (Fig. 2A, Top). This is consistent with recent clinical trials in which vaccination with AMA-1 (40) or MSP-1 (41) did not confer protective immunity, although reformulating these Abs to CSP may benefit immune responses (36). Of note, Abs to PfAg were significantly higher in protected individuals in the present study than seen among naive volunteers (48), which may reflect the differences in naive and memory B cells (MBCs) and the ability of these Abs to recognize epitopes. Ab reactivity to Pf myosin was associated with protection from malaria. Thus, given how little is known about the infection biology of Pf in humans, it may be premature to dismiss immunogenic proteins categorically as potential vaccine targets based solely on subcellular location.

An inherent drawback to the use of the in vitro transcription and translation system to produce Pf proteins is the possibility that not all proteins will be properly folded and display all possible antigenic epitopes. We observed a correlation between Ab reactivity to proteins expressed in this system and to the corresponding well-characterized correctly folded recombinant proteins spotted on the same array; however, despite this correlation, it is likely that this method will fail to detect all potential Ab reactivities. This analysis also sheds light on the interface between the complex multistage Pf parasite and the host immune response by determining the life cycle stage, subcellular location, and biological process of the Pf antigens recognized by Pf-exposed individuals in this study. Further analysis to determine whether patterns of Ab reactivity against individual antigens can be predicted by these and other proteomic features may inform malaria vaccine development.

This study also provided insight into the kinetics of naturally acquired humoral responses to Pf in an area of intense seasonal malaria transmission. We found that Ab levels to Pf proteins rose dramatically in children during the 6-month malaria season; however, most of this response appeared to be short-lived based on cross-sectional analysis at the end of the 6-month dry season, which revealed only modest incremental increases in Ab levels with age. Because there is little to no Pf transmission at the study site during the 6-month dry season (12, 13) and IgG has a half-life of ~21 days (48), we infer that Pf-specific Abs in circulation at the end of the dry season are generated by long-lived plasma cells (LLPCs), whereas the increase in Ab levels observed after the malaria season likely reflects the differentiation of naive and memory B cells (MBCs) into short-lived plasma cells (SLPCs) that disappear by the end of the next dry season. Thus, it appears that the Pf-specific LLPC compartment gradually “fills” with repeated Pf exposure. In age-adjusted analysis, protection from malaria was associated with the overall level of Pf-specific Abs in circulation before the malaria season, whereas Ab levels after the malaria season were not. Taken together, these data suggest that the delayed acquisition of immunity to uncomplicated malaria may be attributable, in part, to the gradual stepwise acquisition of LLPCs, not conferring protection until a critical threshold of circulating Ab is exceeded. Our results also suggest that SLPCs derived from MBCs in response to acute Pf infection, a process that peaks 6–8 days after antigen reexposure (49), at least in the case of vaccination, may not provide Ab rapidly enough to prevent the onset of malaria symptoms, which can occur as early as 3 days after the start of the blood stage infection (50). This model stands in contrast to the humoral immune response after reexposure to some viruses in which longer incubation periods allow virus-specific MBCs to differentiate into plasma cells that contribute to the control of viral replication before symptoms develop. For example, follow-up studies of hepatitis B vaccinees have shown that protection can persist despite the decline of anti-hepatitis B Abs to undetectable levels (51), presumably because of the recall response of persistent MBCs. An alternative explanation for the maintenance of Ab levels through the 6-month dry season is the persistence of low-grade parasitemia despite little to no Pf transmission at the study site. At present, the molecular and cellular mechanisms that underlie the generation and maintenance of Pf-specific LLPCs and MBCs are not known. It is possible that the large number of Pf antigens overwhelms the immune system’s capacity to select for and commit a sufficient number of MBCs and plasma cells specific for any given Pf antigen to a long-lived pool (52). If immunity to clinical malaria requires high levels of Abs to a large number of Pf proteins, the inability to commit large numbers of LLPCs to differentiate into plasma cells may attribute to the control of viral replication before symptoms develop. For example, follow-up studies of hepatitis B vaccinees have shown that protection can persist despite the decline of anti-hepatitis B Abs to undetectable levels (51), presumably because of the recall response of persistent MBCs. An alternative explanation for the gradual acquisition of malaria immunity is the length of time needed to acquire Abs that cover the range of antigenic diversity in the parasite population (53).

This study demonstrates how protein microarrays representing large portions of the Pf proteome can be used to probe the complex interface between the parasite and the host immune response and to identify Ab profiles against known and hypothetical proteins that are associated with naturally acquired malaria immunity. This approach, if validated in larger studies and in other epidemiological settings, could prove to be a useful strategy for identifying malaria vaccine targets and for better understanding fundamental properties of the human immune response to Pf.

Methods
Ethics Statement. The Ethics Committee of the Faculty of Medicine, Pharmacy, and Odonto-Stomatology and the Institutional Review Board at the National Institute of Allergy and Infectious Diseases, National Institutes of Health, approved this study. Written informed consent was obtained from adult participants and from the parents or guardians of participating children.

Study Cohort and Clinical End Points. This study was carried out in Kambia, Mali. A detailed description of the study site and cohort design has been reported elsewhere (12). Of note, blood smears were examined to detect asymptomatic Pf infection at study enrollment and at scheduled follow-up visits every 2 months during the 6-month malaria season. Other features of the cohort study design are highlighted in Results.
Protein Microarray Chip Fabrication, Ab Profiling, and Data Normalization Procedure.

Protein microarray chips consisting of 2,320 Pf proteins (representing 1,204 unique proteins) were fabricated as described previously (6) (SI Text). The Ab profiling and data normalization procedures are described in SI Text.

Data Analysis.

Data analysis methods are described in SI Text.

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