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Naturally Acquired Immune Responses to *Plasmodium falciparum* Sexual Stage Antigens Pfs48/45 and Pfs230 in an Area of Seasonal Transmission▽

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Acquisition of immunity to *Plasmodium falciparum* sexual stages is a key determinant for reducing human-mosquito transmission by preventing the fertilization and the development of the parasite in the mosquito midgut. Naturally acquired immunity against sexual stages may therefore form the basis for the development of transmission-blocking vaccines, but studies conducted to date offer little in the way of consistent findings. Here, we describe the acquisition of antigametocyte immune responses in malaria-exposed individuals in Burkina Faso. A total of 719 blood samples were collected in a series of three cross-sectional surveys at the start, peak, and end of the wet season. The seroprevalence of antibodies with specificity for the sexual stage antigens Pfs48/45 and Pfs230 was 2-fold lower (22 to 28%) than that for an asexual blood stage antigen glutamate-rich protein (GLURP) (65%) or for the preerythrocytic stage antigen circumsporozoite protein (CSP) (54%). The youngest children responded at frequencies similar to those for all four antigens but, in contrast with the immune responses to GLURP and CSP that increased with age independently of season and area of residence, there was no evidence for a clear age dependence of responses to Pfs48/45 and Pfs230. Anti-Pfs230 antibodies were most prevalent at the peak of the wet season ($P < 0.001$). Our findings suggest that naturally acquired immunity against Pfs48/45 and Pfs230 is a function of recent exposure rather than of cumulative exposure to gametocytes.

Malaria transmission depends on the presence of infectious sexual stage parasites in human peripheral blood, and naturally acquired immune responses to these or other stages can affect malaria transmission in several ways. An important indirect manner in which they influence malaria transmission is by reducing the number of asexual parasites that are the source of gametocytes. Immune responses can also influence malaria transmission more directly. Antibodies with specificity for sexual stages have been associated with a reduction of *Plasmodium falciparum* gametocyte prevalence in semi-immune individuals living in an area of Irian Jaya, Indonesia, where malaria is hyperendemic (1). Early-stage (stage I and IIa) gametocytes express the parasite protein *P. falciparum* EMP1 (PfEMP1) on the erythrocyte surface (38), while recently identified proteins may be expressed in later developmental stages (38, 46). Immune responses against these gametocyte-derived surface antigens may be related to direct clearance of gametocytes (47) and may explain why the duration of gametocyte carriage appears to decrease with age (8). A third way in which antibody responses can affect transmission is by reducing the infectiousness of gametocytes once ingested by mosquitoes.

Gametocytes in infected erythrocytes and gametes that emerge from erythrocytes inside the mosquito midgut express stage-specific antigens on their surfaces (27, 50). These antigens have a role in the fertilization or sporogonic development of malaria parasites in mosquitoes (43, 44). A proportion of gametocytes die in the human host without being passed on to a mosquito, thereby exposing sexual stage antigens to the human immune system. Sexual stage-specific antibodies may be elicited against these antigens (31) and may play a role in transmission-blocking immunity by preventing fertilization or the development of sporogonic stage parasites in mosquitos (4, 7, 18, 20, 21, 42, 44, 49). These antibody responses may reduce the spread of malaria in human populations. A better understanding of naturally acquired sexual stage immunity is thus

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relevant to malaria control, as it may form the basis for the development of malaria transmission-blocking vaccines.

Pfs230 and Pfs48/45 are major gametocyte and gamete surface antigens that induce antibody responses in naturally exposed individuals (7, 18, 21, 42, 44) that are associated with functional transmission-reducing immunity (21, 25, 42, 44).

Here, we describe the profiles of naturally acquired sexual stage immune responses to Pfs230 and Pfs48/45 in individuals from an area of intense seasonal malaria transmission in Burkina Faso.

MATERIALS AND METHODS

Study site and population. The study was conducted in a region close to Ouagadougou, the capital of Burkina Faso (West Africa), where malaria is endemic. Two areas of different endemicity (16) were included in the study to test the effect of transmission intensity on sexual stage immunity. *Plasmodium falciparum* is the predominant malaria species in the region, accounting for 90% of the infections; the remaining 10% being attributed to *Plasmodium malariae* and *Plasmodium ovale* (24).

The study's participants are members of subsistence farming communities, and all are permanent residents in the area. Participants were explained the procedures, risks, and benefits involved in the study, and their consent was obtained. The study protocol was viewed and received the written approval of the Ministry of Health of Burkina Faso (research authorization number 2000/3174/MS/SG/DEP).

Measurement of transmission intensity. Repeated CDC light trap captures were carried out in both areas to estimate transmission intensity according to both area and season. Each area consisted of 3 grouped villages. Indoor mosquito captures extended from June to November 2002. The CDC light trap was placed close to the bed of the sleeper in randomly selected houses, and mosquito capture was done from 7:00 p.m. to 6:00 a.m. Mosquito species were identified morphologically, counted, and stored in tubes with silica gel. A representative sample of *Anopheles gambiae* mosquito thoraces and heads were examined to obtain a *P. falciparum* circumsporozoite protein (CSP) positivity index using a routine CSP enzyme-linked immunosorbent assay (ELISA) (2). The monthly sporozoite rate was estimated by testing representative samples (approximately 50% of all mosquitoes caught from each trap) of randomly selected specimens of mosquitoes from each village. The entomological inoculation rate (EIR) was calculated as the multiplication sporozoite rate divided by the biting rate.

Blood sample collection. The seasonally spaced cross-sectional surveys for parasitological and serological data collection coincided with entomological data collection in 2002. At each survey, approximately 300 individuals (~150 from each area of residence) were randomly selected from village census lists aiming to include 60 individuals (10 per village) from each of five predefined age groups: 1 to 4, 5 to 9, 10 to 14, 15 to 29, and ≥30 years of age. Individually selected individuals were invited to a sampling point and were systematically included until the required sample sizes were reached.

For parasites counts, a blood slide film was made from finger prick blood of each individual. For specific antiplasmodial-antibody measurements, 500-μl finger prick blood samples were drawn. Plasma was separated by centrifugation and stored at −20°C before use. Subjects with fever (body temperature of ≥37.5°C) were treated with an antimalarial drug (chloroquine [CQ]) according to the national policy for malaria treatment used in 2002.

Microscopic detection of *P. falciparum* parasites. Slides were read independently by two microscopists, each examining 100 microscopic fields, and the mean density was used. A third reader was involved when the first two readers disagreed about the prevalence or when estimated densities differed by ≥30%. In these cases, the mean density of the two closest readings was used. Asexual stage and gametocyte densities were simultaneously assessed by counting parasites within 1,000 leukocytes in the thick smear. The lower limit of microscopy for gametocyte quantification was estimated at 5 parasites/μl of blood. Parasite counts were converted to numbers of parasites per μl by assuming a standard count of 8,000 leukocytes/μl of blood.

Antigens. Plasma IgGs with specificity for Pfs48/45 and Pfs230 antigens derived from an extract of mature *P. falciparum* NF54 strain gametocytes were measured by ELISA (see below). For this purpose, mature gametocytes were produced in an automated static culture system in red blood cells of blood group O^+ and nonimmune AB serum (39) and harvested after 13 to 14 days. Gametocyte purification was as previously described (50). Briefly, mature gametocytes were isolated at 37°C to prevent their activation. Parasite culture was loaded on a cushion of 63% Percoll (GE Healthcare; catalog number 17-0891-01) and centrifuged for 30 min at 1,500 × *g*. The purified gametocytes were then aliquoted and stored at −70°C before use. For extraction of Pfs48/45- and Pfs230-enriched proteins, gametocytes were resuspended in 1% sodium deoxycholate-Tris-NaCl and 1 mM phenylmethylsulfonyl fluoride (PMSF), incubated for 10 min at room temperature, and spun at 13,000 rpm for 10 min. The supernatant was collected as Pfs48/45- and Pfs230-enriched antigen extract and diluted in 0.25% PBSTM (2.5% milk and 0.05% Tween 20 in phosphate-buffered saline [PBS]) for use. The synthetic peptide $NANP_6$, corresponding to the repeat region of the circumsporozoite protein, and the synthetic peptide GLURP $GMP_{85–213}$ LR67 (48) were used in standardized ELISAs for sporozoite- and asexual blood stage-specific antibody detection, respectively (see below).

Samples selection. Although samples for serology were available from almost all participants, it was not possible to screen all samples for the presence of sexual stage antibodies due to the labor intensiveness and costs involved in the necessary antigen preparation. ELISA was therefore performed on a representative subsample of randomly selected samples. For this purpose, samples were randomly selected from the list of available samples for each age group and season separately. Initially, a number of 150 samples per season, i.e., 25 samples from individuals 1 to 4 and 5 to 9 years of age and 50 samples from individuals 10 to 19 and ≥20 years of age, were randomly selected for testing both Pfs48/45 and Pfs230 antigens. Samples sizes were approximately similar for both antigen tests at the start of the wet season (137 for Pfs48/45 and 136 for Pfs230), the peak of the wet season (149 and 148, respectively), and the end of the wet season (125 and 129, respectively). To exclude possible variation in gametocyte antigen preparations, only samples that were concurrently tested for both Pfs48/45 and Pfs230 antigens extracted from the same batch of gametocytes were used for data analysis. This resulted in a total of 130 samples at the start and end of the wet season and 150 at the peak of the wet season, explaining the slight inconsistencies in the sample sizes between seasons, age groups, and areas of different levels of endemicity.

ELISA. The presence of anti-Pfs48/45 and anti-Pfs230 IgG antibodies in plasma samples was determined by coating in the wells of 96-well polystyrene U-bottom hard ELISA plates (Sterilin; International Medical Products B.V., Zutphen, The Netherlands) with 10 μg/ml of anti-Pfs48/45 rat monoclonal antibody (MAb) 85RF45.3 (45) or anti-Pfs230 rat MAb 63F6D7-F(ab)$_2$ (41), diluted in PBS. Free sites were blocked with 5% milk (Marvel; Premier International Foods Ltd., Spalding, Lincs, United Kingdom) in PBS, and Pfs48/45 and Pfs230 antigens contained in 50 μl gametocyte extract (250,000 gametocyte equivalents/well) were captured by overnight incubation at 4°C. The buffer (0.25% PBSTM) used for dilution of gametocyte extract was added to control wells (i.e., no extract added) for background measurement. A dilution (1:100) of the test plasma in 0.25% PBS-TM was added to the wells prepared with and without antigen (control wells) and incubated for 2 h at room temperature. The plate was washed, and bound IgG antibodies were detected with the addition of 100 μl of 1:30,000-diluted goat anti-human IgG–peroxidase (H+L; Pierce) for 1 h, 30 min at room temperature. Wells were washed with PBS and subsequently incubated with tetramethyl benzidine (TMB) substrate solution for 20 min. The color reaction was stopped with 4 N H_2SO_4, and the optical density (OD) was read at 450 nm in an Anthos 2001 microplate reader (Labtec B.V.). All plasma samples were tested in duplicate. Three nonimmune plasma samples from Dutch blood bank donors as negative controls and one positive-control plasma of a Dutch man that had been exposed to malaria for almost 30 years in sub-Saharan Africa were included per plate. The value for IgG titer (OD) of a sample was expressed as the difference in OD between the antigen and control wells. The cutoff was calculated as the mean OD of negative controls plus 2 standard deviations. A sample was considered positive if its background-adjusted OD was above the cutoff.

GLURP and $NANP_6$ IgG ELISA. To evaluate anti-asexual blood stage or antisporozoite antibody responses, flat-bottom high-binding 96-well microtiter plates (Nunc MaxiSorp; Nalge Nunc International Corp., Life Technologies, The Netherlands) were coated with 0.2 μg/ml of $GLURP_{85–213}$ in 0.05 M carbonate buffer (50 μl/well) or 1 μg/ml of $NANP_6$ in PBS (50 μl/well) at 4°C. Coated plates were incubated overnight at 4°C and washed with PBS-Tween 20 (PBST). Free sites were blocked with 150 μl/well of 2.5% milk-PBS (Marvel). Subsequently, the blocker was washed off and plates were incubated for 1 h at room temperature with 50 μl of 1:200-diluted plasma in PBSTM for GLURP ELISA or with 50 μl of 1:100-diluted plasma for antisporozoitic ELISA. Plates were washed and incubated for 1 h with rabbit anti-human IgG-peroxidase (P-214; Dako) diluted 1:10,000 in PBSTM before reaction with the substrate (TMB). The staining was stopped after 15 min of reaction with 4 N H_2SO_4, and the plate was read as described for the Pfs48/45-Pfs230 ELISA. Plasma was considered positive if the

OD value was greater than 2 standard deviations above the mean of results for the negative-control plasma samples from Dutch blood bank donors.

Statistical analysis. Study participants were categorized into groups by age (1 to 4 years, 5 to 9 years, 10 to 19 years, and ≥20 years). The statistical analysis was performed using SPSS version 14.0 (SPSS Inc., Chicago, IL). The influence of age on antibody prevalence was tested using logistic-regression analyses and age categories. Multivariate regression models allowed for confounding effects of age, season, and area of residence. The Pearson χ^2 test was used for comparing proportions and trends in dichotomous variables. Spearman's rank correlation test was used to assess association between antibody levels of tested antigens. The association between Pfs230 and Pfs48/45 antibody responses and functional transmission-reducing activity (TRA) in the standard membrane feeding assay (SMFA) in previous studies (9, 11, 18) was presented after categorization of TRA as >50% and >90% reduction (9, 11, 18) and calculating odds ratios (OR) with 95% confidence intervals (95% CI). The level of significance was set at a two-tailed P value of <0.05.

RESULTS

Entomology. A representative sample of 4,525 mosquitoes was tested for transmission intensity estimates. EIR details per season and area of residence are summarized in Table 1. Overall, the mean EIR was estimated to be 28.5 infective bites/person/month (ib/p/m) in the whole study area and this varied by season (1.47, 69.57, and 14.67 ib/p/m at the start, peak, and end of the wet season, respectively) and area of residence (52.4 ib/p/m versus 3.05 ib/p/m).

Parasitology. A total of 719 blood slide samples were collected over the three cross-sectional surveys. At the site with the highest endemicity, 147, 101, and 130 samples were collected at the start, peak, and end of the wet season, respectively. At the site of lower endemicity, these figures were 149, 50, and 142, respectively. The overall proportion of individuals harboring asexual parasites was 63.4% (456/709), while 18.9% (136/709) carried gametocytes. There were significant age-dependent decreases both in the prevalences of asexual parasites (OR = 0.54; $P < 0.001$; 95% CI, 0.46 to 0.63) and gametocytes (OR = 0.61; $P < 0.001$; 95% CI, 0.51 to 0.74) and in the densities of asexual parasites [$\beta = -0.027$, se(β) = 0.003; $P < 0.001$] and gametocytes [$\beta = -0.009$, se(β) = 0.022; $P = 0.004$] after adjustment for season and area of residence.

Parasite prevalence and density showed seasonal fluctuations. The prevalence of asexual parasites was significantly higher at the peak than at the start ($P < 0.001$) and the end ($P = 0.004$) of the wet season. Similarly, we observed a higher asexual parasite density at the peak than at the start ($P < 0.001$) and the end ($P = 0.01$) of the wet season. Details on gametocyte prevalence are presented in Table 1. As with asexual parasites, gametocytes were more prevalent at the peak (28.5%; 43/151 samples) than at the start (22%; 65/296 samples) ($P = 0.1$) of the wet season and were least prevalent at the end (10.3%; 28/272 samples) ($P < 0.001$) of the wet season. The median density of gametocytes/μl blood was higher at the peak (24 parasites; interquartile range [IQR], 24 to 72 parasites) than at the start (24 parasites; IQR, 16 to 40 parasites) ($P = 0.03$) and, at a borderline level of significance, lower than at the end (40 parasites; IQR, 40 to 57 parasites) ($P = 0.09$) of the wet season. Despite a substantial difference in EIR between them, there was no significant variation in asexual parasite and gametocyte prevalences between the study areas. The prevalence of asexual parasites was 61% (208/341 samples) in the low-transmission area and 65.6% (248/378 samples) in the high-transmission area ($P = 0.2$). The distribution of gameto-

TABLE 1. Entomological inoculation rates and gametocyte indices in the study population

Wet season	No. of traps		EIR (95% CI)		% gametocyte prevalence (n/N) in patients[a]								Total
					1–4 yr		5–9 yr		10–19 yr		20+ yr		
	Low endemicity	High endemicity	Low endemicity	High endemicity	Low endemicity	High endemicity	Low endemicity	High endemicity	Low endemicity	High endemicity	Low endemicity	High endemicity	
Start	36	36	0.0	2.9	20.0 (6/30)	43.3 (13/30)	30.0 (9/30)	34.5 (10/29)	17.3 (9/52)	26.5 (13/49)	2.7 (1/37)	10.3 (4/39)	22.0 (65/296)
Peak	51	50	9.16	129.9	40.0 (4/10)	42.9 (9/21)	40.0 (4/10)	36.8 (7/19)	42.1 (8/19)	22.9 (8/35)	9.1 (1/11)	7.7 (2/26)	28.5 (43/151)
End	54	53	0.0	29.3	24.0 (6/25)	14.8 (4/27)	9.7 (3/31)	6.9 (2/29)	13.7 (7/51)	5.9 (2/34)	5.7 (2/35)	5.0 (2/40)	10.3 (28/272)

[a] n, number of observations with a positive result; N, total number of observations.

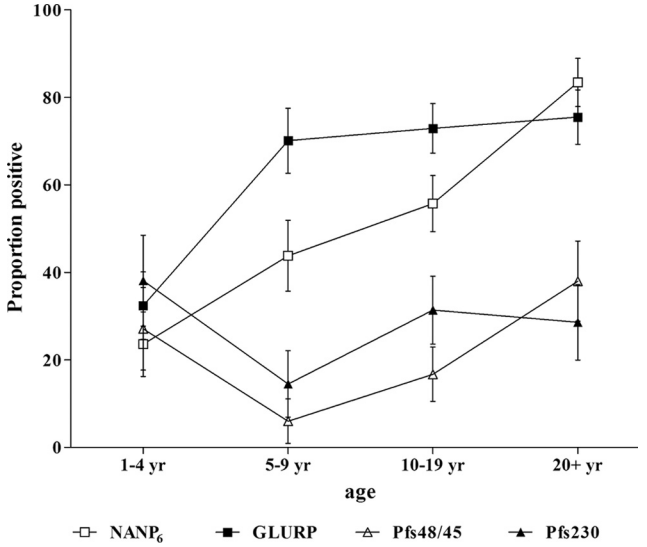

FIG. 1. Prevalence of antibodies against preerythrocytic stage ($NANP_6$), asexual blood stage (GLURP), and sexual stage (Pfs48/45 and Pfs230) parasites in relation to age. The error bars show the ± limits of the 95% confidence intervals. The numbers of plasma samples tested per age group (1 to 4, 5 to 9, 10 to 19, and ≥20 years of age) are 127, 144, 230, and 175 for $NANP_6$; 142, 147, 236, and 184 for GLURP; 85, 84, 138, and 108 for Pfs48/45; and 84, 83, 137, and 105 for Pfs230, respectively.

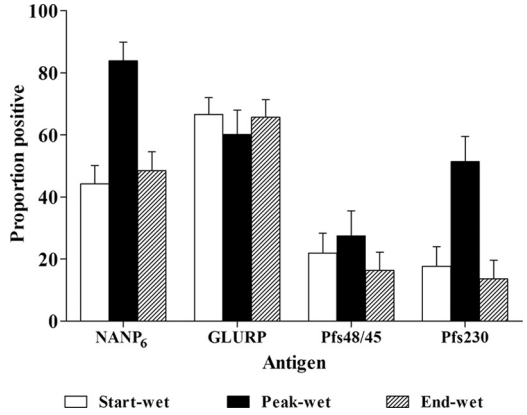

FIG. 2. Prevalence of antibodies against $NANP_6$, GLURP, Pfs48/45, and Pfs230 in relation to season. Bars indicate the proportion of positive individuals in each area of residence. Error bars indicate the upper limit of the 95% confidence interval around the proportion. The numbers of individuals (with antibodies) during the different seasons were as follows: at the start of the wet season, 119 (of 269) for $NANP_6$, 195 (of 293) for GLURP, 30 (of 137) for Pfs48/45, and 24 (of 136) for Pfs230; at the peak of the wet season, 120 (of 143) for $NANP_6$, 89 (of 148) for GLURP, 41 (of 149) for Pfs48/45, and 76 (of 148) for Pfs230; and at the end of the wet season, 128 (of 264) for $NANP_6$, 92 (of 176) for GLURP, 17 (of 125) for Pfs48/45, and 21 (of 129) for Pfs230.

cytes in both areas is shown in Table 1. The proportions of gametocyte carriers were 17% (60/341 samples) in the low-transmission area and 20.1% (76/378 samples) in the high-transmission area ($P = 0.4$). This equality in parasite prevalence may reflect the high endemicity of malaria across the entire area (16).

Serology. Plasma samples were screened for antibody response profiles in the population and related to age (Fig. 1), season (Fig. 2), and area of residence (Fig. 3). The average prevalence of IgG antibodies was 54.3% (367/676 samples) for the sporozoite antigen $NANP_6$ and 64.9% (460/709 samples) for the asexual blood stage antigen GLURP. In contrast to sporozoite and asexual blood stage antigens, sexual stage-specific antibody responses were detected only in a minority of the samples: 22.2% (92/415 samples) contained anti-Pfs48/45 IgG and 28.6% (117/409 samples) anti-Pfs230 IgG.

Effect of age on antibody responses. Among children below 5 years of age, the prevalences of antibodies with specificity for $NANP_6$ (23.6%) and GLURP (32.4%) were broadly similar to those for Pfs48/45 (27.1%) and Pfs230 (38.1%). As expected, the prevalences of asexual stage antibodies to $NANP_6$ and GLURP increased significantly with age (adjusted OR = 2.35, $P < 0.001$, and 95% CI of 1.98 to 2.78, and adjusted OR = 1.79, $P < 0.001$, and 95% CI of 1.53 to 2.08, respectively), reflecting cumulative exposure to infection, while no evidence of an age-dependent increase in sexual stage-specific antibody responses was observed (Table 2). The seroprevalences for Pfs48/45 and Pfs230 in the youngest children (27.1% and 38.1%, respectively) were comparable to those in adults (38% and 28.6%, respectively). However, there was a significant decline of Pfsf48/45- and Pfs230-specific antibody prevalence in 1 to 4 year olds compared to 5 to 9 year olds ($\chi^2 = 13.61$, $P = 0.001$, and $\chi^2 = 12.02$, $P < 0.001$, respectively), followed subsequently by a significant increase with increasing age (OR = 3.09, $P < 0.001$, and 95% CI of 2.02 to 4.71, and OR = 1.41, $P = 0.04$, and 95% CI of 1.01 to 1.97, respectively) (Fig. 1).

Differences between seasons and areas. GLURP antibody responses showed no variation according to season or area,

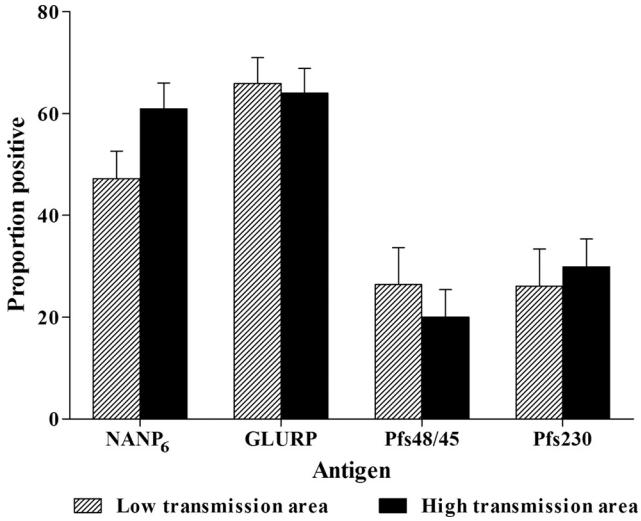

FIG. 3. Prevalences of antibodies against $NANP_6$, GLURP, Pfs48/45, and Pfs230 in relation to the area of residence. Bars indicate the proportion of positive individuals in each area of residence. Error bars indicate the upper limit of the 95% confidence interval around the proportion. The numbers of positive individuals in both areas of residence were as follows: in the high-transmission area, 213 (of 350) for $NANP_6$, 238 (of 372) for GLURP, 55 (of 275) for Pfs48/45, and 81 (of 271) for Pfs230, and in the low-transmission area, 154 (of 326) for $NANP_6$, 222 (of 337) for GLURP, 37 (of 140) for Pfs48/45, and 36 (of 138) for Pfs230.

while, conversely, the prevalence of $NANP_6$ antibodies differed by both season (Fig. 2) and area of residence (Fig. 3). Antibodies with specificity for $NANP_6$ were detected in 83.9% of individuals at the peak of the wet season, while this proportion was significantly lower at the start (44.2%) and at the end (48.5%) of the wet season. Thus, the prevalence of $NANP_6$ antibodies at the peak of the wet season was significantly higher than either at the start ($\chi^2 = 60.34$; $P < 0.001$) or at the end ($\chi^2 = 48.91$; $P < 0.001$) of the wet season. There was also a difference in $NANP_6$ antibody prevalence between the area of high transmission (60.9%) and the area of lower transmission (47.2%; $\chi^2 = 12.61$; $P < 0.001$).

Prevalences of sexual stage-specific antibodies in relation to season. Pfs48/45 antibody prevalence increased at the peak of the wet season (27.5%) but did not significantly differ from the prevalence at either the start (21.9%) or at the end of the wet season (16.3%) (Fig. 2). The variation in Pfs230-specific antibody prevalence was significant, reaching 51.4% at the peak compared to 17.6% at the start ($\chi^2 = 35.29$; $P < 0.001$) and 13.6% at the end ($\chi^2 = 42.99$; $P < 0.001$) of the wet season. This increased Pfs230 antibody prevalence at the peak of the wet season remained significant after an adjustment for age (adjusted OR = 4.93; $P < 0.001$; 95% CI, 2.85 to 8.52) (Table 2). Both antigens are on the surface of the gametocyte and were expected to have shown similar patterns of immune response. The difference observed in their immune responses may be dependent on the difference in their immunogenicities, with Pfs230 being more immunogenic than Pfs48/45 (22).

In terms of area of residence, there was no difference in the prevalences of antibodies with specificity for either Pfs48/45 or Pfs230 (Table 2).

The relationship between antibody responses and parasite carriage is shown in Table 2. Both anti-$NANP_6$ and anti-GLURP immune responses were significantly higher in individuals negative for gametocytes than in individuals positive for gametocytes (adjusted OR = 0.62, $P = 0.04$, and 95% CI of 0.38 to 1.00 for $NANP_6$, and adjusted OR = 0.58, $P = 0.01$, and 95% CI of 0.38 to 0.90 for GLURP). The seroprevalences for Pfs48/45 and Pfs230 antibodies were not influenced by the concurrent presence of asexual parasites or gametocytes.

Correlation of antibody responses between the different antigens. We also examined the correlation between antibody responses at the individual level. As presented in Table 3, the levels of antibody directed to $NANP_6$ and Pfs230 and those to Pfs230 and Pfs48/45 were strongly correlated ($P \leq 0.001$.) Antibody levels to GLURP were associated with those to $NANP_6$.

Functionality of sexual stage immune responses. The volume of plasma that remained from this study was insufficient to study functional transmission-reducing activity in the standard membrane feeding assay (SMFA). To aid interpretation of the current results, paired ELISA-SMFA data from three studies (9, 11, 18) were combined and are presented in Table 4. Sexual stage antibody responses were determined using exactly the same ELISA methodology as used in the current study. The TRA of >50% and TRA of >90% were significantly associated with antibodies to Pfs48/45 ($P < 0.001$ and $P < 0.001$, respectively) and Pfs230 ($P < 0.007$ and $P < 0.002$, respectively).

TABLE 2. Factors associated with antibody prevalence to *P. falciparum* pre-erythrocytic and asexual blood stage antigens and sexual stage antigens[a]

Parameter	Factor	No. of patients, $NANP_6$ univariate OR (95% CI)	No. of patients, $NANP_6$ multivariate OR (95% CI)	No. of patients, GLURP univariate OR (95% CI)	No. of patients, GLURP multivariate OR (95% CI)	No. of patients, Pfs48/45 univariate OR (95% CI)	No. of patients, Pfs48/45 multivariate OR (95% CI)	No. of patients, Pfs230 univariate OR (95% CI)	No. of patients, Pfs230 multivariate OR (95% CI)
Age	1–4 yr	127, 1 (ref)	127, 1 (ref)	142, 1 (ref)	142, 1 (ref)	85, 1 (ref)	85, 1 (ref)	84, 1 (ref)	84, 1 (ref)
	5–9 yr	144, 2.51 (1.48–4.25)	144, 2.57 (1.51–34.38)	147, 4.88 (2.96–8.04)	147, 4.88 (2.96–8.04)	84, 0.17 (006–0.47)	84, 0.16 (0.06–0.47)	83, 0.27 (0.12–0.58)	83, 0.27 (0.12–0.58)
	10–19 yr	230, 4.05 (2.49–6.59)	230, 4.29 (2.62–7.03)	236, 5.60 (3.56–8.82)	236, 5.59 (3.55–8.80)	138, 0.53 (0.28–1.03)	138, 0.52 (0.26–1.00)	137, 0.74 (0.42–1.31)	137, 0.74 (0.41–1.31)
	20+ yr	175, 16.27 (9.19–28.82)	175, 17.16 (9.61–30.62)	184, 6.44 (3.96–10.48)	184, 6.46 (3.97–10.51)	108, 1.65 (0.89–3.05)	108, 1.67 (0.89–3.11)	105, 0.65 (0.35–1.19)	105, 0.64 (0.34–1.18)
Season	Start of wet season	269, 1 (ref)	269, 1 (ref)	293, 1 (ref)	293, 1 (ref)	137, 1 (ref)	137, 1 (ref)	136, 1 (ref)	136, 1 (ref)
	Peak of wet season	143, 6.57 (3.96–10.91)	143, 10.59 (5.91–18.98)	148, 0.75 (0.50–1.14)		149, 1.35 (0.78–2.32)		148, 4.92 (2.85–8.50)	148, 4.93 (2.85–8.52)
	End of wet season	264, 1.18 (0.84–1.66)	264, 1.33 (0.90–1.96)	268, 0.96 (0.67–1.36)		129, 0.69 (0.37–1.28)		125, 0.73 (0.37–1.44)	125, 0.73 (0.37–1.45)
Area	Low tran.	326, 1 (ref)	326, 1 (ref)	337, 1 (ref)		140, 1 (ref)		138, 1 (ref)	
	High tran.	350, 1.73 (1.27–2.35)	1.93 (1.38–2.71)	372, 0.92 (0.67–1.25)		275, 0.69 (0.43–1.12)		271, 1.20 (0.76–1.91)	
Asexual parasites	Absent	244, 1 (ref)		259, 1 (ref)		131, 1 (ref)		128, 1 (ref)	
	Present	432, 0.72 (0.53–0.99)		450, 1.08 (0.79–1.49)		284, 0.65 (0.40–1.05)		281, 0.98 (0.62–1.55)	
Gametocytes	Absent	544, 1 (ref)		575, 1 (ref)	575, 1 (ref)	326, 1 (ref)		321, 1 (ref)	
	Present	132, 0.57 (0.39–0.84)	132, 0.62 (0.38–1.00)	134, 0.52 (0.35–0.76)	134, 0.58 (0.38–0.90)	89, 0.72 (0.40–1.31)		88, 1.06 (0.63–1.78)	

[a] tran., transmission intensity; seroreactivity related to a given variable was adjusted for all other variables that play a significant role primarily in the univariate model. ref, reference group.

TABLE 3. Spearman's rank correlation of levels of antisporozoite and anti-asexual parasite blood stage antigens with the level of anti-sexual parasite stage antigen[a]

Antigen	No. of patients, $NANP_6$ r (P value)	No. of patients, GLURP r (P value)	No. of patients, Pfs48/45 r (P value)	No. of patients, Pfs230 r (P value)
$NANP_6$		671, 0.209 (<0.001)	390, 0.53 (0.29)	384, 0.170 (0.001)
GLURP			413, −0.067 (0.17)	407, −0.039 (0.43)
Pfs48/45				409, 0.414 (<0.001)
Pfs230				

[a] r, Spearman's correlation coefficient. Paired $NANP_6$-GLURP, $NANP_6$-Pfs230, and Pfs48/45-Pfs230 data showed strong correlations ($P \leq 0.001$).

DISCUSSION

As in previous studies (6, 9, 18, 23, 26, 43, 44), we found evidence for naturally acquired immunity against *P. falciparum* sexual stages. Overall, 22 to 28% of our samples were positive for sexual stage-specific antibodies, while 54 to 65% were positive for preerythrocyte- and asexual blood stage-specific antibodies. Unlike with immune responses to $NANP_6$ and GLURP, we found no evidence for age as a predictor for anti-Pfs48/45 and anti-Pfs230 antibody responses. Our data reveal that sexual stage-specific antibody responses increase markedly during the transmission season. This indicates a role for recent exposure to parasite antigens in the short-term boosting of sexual stage-specific immunity, although this requires confirmation in longitudinal studies.

The lower prevalence of sexual stage-specific antibodies than that of either preerythrocyte- or asexual stage-specific antibodies in the same study population confirms previous findings (6, 13, 18). In contrast to antibodies directed to sexual stage antigens, those directed to an asexual blood stage antigen (GLURP) increased with age, as described previously (14, 15, 17, 19, 32), with no apparent season-dependent changes (17, 19). This suggests that anti-GLURP antibody responses may be long-lived and stable, even when antigen exposure decreases in the low-transmission season and/or with increasing age. If sexual stage-specific antibody responses persist in the absence of boosting, we would expect to see a similar pattern of increasing antibody prevalence with increasing age, but despite a significant increase in the prevalence of sexual stage-specific antibodies from 5 to 9 years of age onward in our population, the close similarity of the prevalences in the youngest children and in adults above 20 years of age effectively obscured any such association.

A single recent study reported an age-dependent increase of sexual stage-specific antibody responses, although it should be noted that that study concerned pre- and posttreatment determinations in residents of an area with a very low rate of transmission and is therefore not directly comparable with the study that we report here (11). The results of other studies of the age dependence of such responses are inconsistent (6, 18, 30). In Cameroon, the level of transmission-blocking immunity, as measured in membrane feeding experiments, was not related to age (3, 5). The lack of age dependence of sexual stage-specific antibody responses has often been attributed to their short-lived nature and hence a requirement for frequent boosting (30, 40). Cumulative immune memory, likely the result of repeated exposure to infections, is thought to form the basis of the age-dependent increase of asexual blood stage-specific immune responses (51). Immature and/or short-term memory for responses directed to sexual stage antigens is plausible (30) and possibly explained by a predominantly T-cell-independent induction of the immune response (29).

Interestingly, the prevalence of sexual stage-specific IgG responses in the youngest children in our study group was similar to those of the asexual (GLURP) and preerythrocytic ($NANP_6$) antigens, suggesting that sexual stage-specific immune responses may readily develop in response to antigen exposure early in life. Since age-related cumulative exposure seems to have little effect on sexual stage immune responses, we speculate that the greater prevalence in young children may reflect the initial immune response to gametocytes, as was observed in individuals after a single or limited number of exposures to infection with either *P. falciparum* (6, 22, 33) or *Plasmodium vivax* (31). The remarkable nadir in both Pfs48/45 and Pfs230 seroprevalences in children aged 5 to 9 years old was previously observed in populations exposed to endemic malaria. The studies found that sexual stage immune responses decreased with age in the younger age groups but that there was no further decline in older age groups (6, 18). Sexual stage antibody responses in young children are likely to be the result of high gametocyte exposure in this age group. In older chil-

TABLE 4. Association between sexual stage antibody prevalence and functional transmission-reducing activity in three studies using the same ELISA methodology[a]

Functional transmission-reducing activity	Sexual stage antibody response			
	Pfs48/45 antibody prevalence (n = 350)		Pfs230 antibody prevalence (n = 344)	
	OR (95% CI)	P value	OR (95% CI)	P value
>50% reduction	3.76 (2.27–6.23)	<0.001	1.87 (1.18–2.96)	0.007
>90% reduction	6.08 (3.21–11.49)	<0.001	2.84 (1.47–5.50)	0.002

[a] This summary table combines data from Tanzania and Indonesia (9, 11, 18) from studies that used identical ELISA protocols and that related antibody prevalence to functional transmission-reducing activity (i.e., the percentages of reduction in mosquito oocyst numbers in test samples compared with those of controls) in standard membrane feeding assays.

dren, gametocyte exposure decreases, possibly explaining the reduction in antibody prevalence. In adults, gametocyte exposure may be lowest, but sexual stage commitment during infections may be relatively increased (34) and antibody responses to sexual stage antigens may become more long-lived, reflecting a maturation of the immune response.

An important finding from the study reported here is that sexual stage-specific antibody responses may vary with season. Pfs230-specific antibody prevalence, in particular, increased during the peak of the transmission season, suggesting that the level of sexual stage-specific antibodies may reflect recent exposure. A possible association between sexual stage immunity and recent antigen exposure is also suggested by the correlation between $NANP_6$ and anti-sexual stage antibodies. In contrast to those directed to GLURP, $NANP_6$-specific antibody responses seemed to be short-lived. Thus, the pattern of variation in $NANP_6$ antibody responses closely reflected the seasonality of transmission in our study, indicating that seroreactivity to $NANP_6$ is dependent on recent exposure and therefore highly susceptible to changes in sporozoite exposure. The close association that we observed between $NANP_6$- and Pfs230-specific antibodies may be an indication that the immune responses to sexual stage antigens are therefore also related to recent exposure to infection, explaining the seasonality of the anti-Pfs230 antibody response. Our results showed that many infections with asexual parasites are accompanied by gametocytes (35, 37), and this proportion is further increased if submicroscopic gametocyte densities are considered (10). Since asexual parasite carriage increases during the transmission season (36), exposure to gametocytes increases in parallel, possibly explaining the seasonality of anti-sexual stage IgG responses. However, the seasonality of sexual stage-specific immune responses (28) has been rarely studied, making any comparison difficult. The importance of recent exposure to infection for the acquisition of sexual stage-specific immunity has been reported from previous studies in which the prevalence of anti-Pfs48/45 and anti-Pfs230 antibodies increased in migrants from an area of nonendemicity to an area of endemicity (9) and where functional transmission-reducing immunity was shown to increase during the transmission season (12). We did not determine functional transmission-reducing immune responses in the current data set. Three studies that used the same methodology for determining total IgG antibody responses to Pfs48/45 and Pfs230 did relate these responses to the TRA in the standard membrane feeding assay (9, 11, 18). When combined, these data show a strong association between Pfs48/45 and Pfs230 antibody prevalence and different levels of TRA. These data support the current findings on sexual stage antibody responses that plausibly reflect associations between functional TRA and age, season, and recent exposure to malaria antigens.

We further tried to explore the relevance of recent exposure for the acquisition of parasite stage-specific immunity by relating antibody responses to concurrent asexual parasite and gametocyte carriage. Our results showed that $NANP_6$- and GLURP-specific antibodies appear to negatively associate with gametocytes, suggesting that immune responders to these two antigens are more likely than nonresponders to have a short period of asexual parasite carriage that will translate into a lower likelihood of gametocyte carriage. However, we found no effect of anti-Pfs48/45 or anti-Pfs230 antibodies on gametocyte carriage. Concurrent gametocytes may be positively related to the development of sexual stage-specific immune responses (11), although a straightforward relationship is not always evident (6, 18). Immune responses to gametocyte antigens induced prior to sampling may persist for several weeks after gametocyte clearance (11) or following the reduction of gametocytemia to submicroscopic levels, while acquisition/boosting of responses to gametocytes circulating at the time of sampling may take longer, e.g., after their death/destruction and subsequent clearance. Intuitively, then, an antibody response that is boosted by recent gametocyte antigen exposure can better be determined in studies that determine gametocyte carriage repeatedly at both the microscopic and the submicroscopic level and that relate sexual stage-specific antibody responses to previous as well as current gametocyte exposure.

In summary, plasma samples from malaria-exposed individuals were analyzed to provide indications of the development of naturally acquired immunity to *P. falciparum* sexual stages. Our findings indicate that sexual stage-specific immune responses are naturally acquired in the study population and that they are a function of recent exposure rather than cumulative exposure to gametocytes. This occurs after limited exposure to gametocytes in the youngest age groups, but boosting is also evident during the peak of the transmission season. A next step would be to determine the functional importance of sexual stage-specific antibody responses in this population, which, despite developing both asexual and sexual stage-specific antibody responses, is repeatedly exposed to intense transmission (35, 36).

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We declare that no competing interests exist.

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