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Influence of infection on malaria-specific antibody dynamics in a cohort exposed to intense malaria transmission in northern Uganda

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SUMMARY

The role of submicroscopic infections in modulating malaria antibody responses is poorly understood and requires longitudinal studies. A cohort of 249 children ≤5 years of age, 126 children between 6 and 10 years and 134 adults ≥20 years was recruited in an area of intense malaria transmission in Apac, Uganda and treated with artemether/lumefantrine at enrolment. Parasite carriage was determined at enrolment and after 6 and 16 weeks using microscopy and PCR. Antibody prevalence and titres to circumsporozoite protein, apical membrane antigen-1 (AMA-1), merozoite surface protein-1 (MSP-1$_{19}$), merozoite surface protein-2 (MSP-2) and Anopheles gambiae *salivary gland protein 6 (gSG6) were determined by ELISA.* Plasmodium falciparum *infections were detected in 38·1% (194/509) of the individuals by microscopy and in 57·1% (284/493) of the individuals by PCR at enrolment. Antibody prevalence and titre against AMA-1, MSP-1$_{19}$, MSP-2 and gSG6 were related to concurrent (sub-)microscopic parasitaemia. Responses were stable in children who were continuously infected with malaria parasites but declined in children who were never parasitaemic during the study or were not re-infected after treatment. These findings indicate that continued malaria infections are required to maintain antibody titres in an area of intense malaria transmission.*

Keywords *antibody, epidemiology, humoral immunity, malaria, PCR, submicroscopic*

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INTRODUCTION

Inhabitants of areas with stable malaria transmission develop clinical and parasitological immunity after repeated exposure to *Plasmodium falciparum*. In areas exposed to intense malaria transmission, protection against severe life-threatening malaria is acquired early in life after relatively few malaria episodes (1) while protection against mild malaria or asymptomatic infection develops later in life (2,3). Despite many years of research on this topic, it is unclear which antibodies are associated with protection and how their development is influenced by natural exposure. A major problem in the interpretation of field studies is that antibody responses are related to both protection and exposure. While protection against clinical malaria episodes is associated with the breadth and magnitude of antibody responses (4), these antibodies are acquired after exposure to blood-stage infections; individual variation in antibody repertoires and titres therefore also reflects individual variations in malaria exposure (5–7). As cumulative malaria exposure may reduce susceptibility to clinical disease through mechanisms unrelated to the antibodies being studied, interpretation of findings from cross-sectional and even longitudinal studies (8) is complicated and likely explains why antibodies to specific malaria antigens have inconsistent associations with protection and risk of clinical malaria (7,9–11). As expected, the prevalence and/or titre of antibodies is consistently higher in individuals who have microscopically detectable parasites at the time of sampling compared with parasite-free individuals (6,12). Similarly, individuals with submicroscopic infections may have higher antibody prevalences and titres compared with parasite-free individuals (13). These associations are sometimes interpreted as evidence for immune boosting by recent infection. It is, however,

unclear to what extent these associations are explained by the current infection or by historic differences in exposure, because individuals who are parasitaemic at the time of sampling may simply have had a higher cumulative antigen exposure (7).

The aim of this study was to examine the effect of malaria infection patterns on malaria-specific antibody acquisition and dynamics in an all-age cohort exposed to intense malaria transmission. For this purpose, we determined antibody prevalence and titre against a selection of three blood stages, one sporozoite and one mosquito salivary antigen at three time points.

MATERIALS AND METHODS

Study area

The study was conducted in 2010 in the Abedi parish in Apac district, northern Uganda, a rural area situated between Lake Kyoga and the Victoria Nile (latitude 1·985; longitude 32·535). The area has intense malaria transmission with a parasite prevalence of approximately 70% in children under 10 years (14). *Plasmodium falciparum* is the dominant parasite species; *P. malariae and P. ovale* being present in approximately 4, and 9%, respectively, of the infections (15).

Study design and procedure

This study received ethical approval from the ethical review committees of the London School of Hygiene and Tropical Medicine (#5539), the Med Biotech Laboratories in Kampala and the Uganda National Council for Sciences and Technology (UNCST).

We aimed to recruit individuals from three age strata expected to represent individuals without clinical immunity (<5 years, $n = 250$), individuals with clinical but no parasitological immunity (6–10 years, $n = 125$) and individuals with a high degree of both clinical and parasitological immunity (>20 years, $n = 125$). This sample size was based on a previous study where this number of participants was found to be sufficient for a reliable determination of age-related variation in antimalarial antibody prevalence and titre in relation to recent exposure to malaria (14). Exclusion criteria were a weight-for-height or height-for-age Z-score <-3, severe anaemia (Hb < 5·0 g/dL), or the presence of any chronic disease. Excluded individuals were referred to Apac District Hospital for appropriate clinical management.

To recruit the envisaged number of study participants, we mapped all households within 5 km of Abedi Health Centre using T a handheld global positioning system (Garmin eTrex; Garmin International, Inc., Olathe, KS, USA) and performed a census. Households with at least one child from the lowest age stratum and at least one individual from either of the other age strata were eligible for participation and selected based on computer-generated random tables. From each of the selected households, a maximum of one individual per age stratum and two individuals in total were selected, again using computer-generated random tables. We invited 300 eligible households to participate in the study, estimating that this would generate $\geq 120\%$ of the proposed sample size in each age-stratum: 300 children <5 years of age (target number 250), 150 children 6–10 years of age (target number 125) and 150 adults (>20 years, target number 125). Invitees were enrolled on a first-come first-served basis until the sample size was reached.

At enrolment, individuals were clinically assessed to detect malaria infection or other illness and all participants received antimalarial treatment with artemether/lumefantrine (Lonart®; Bliss Gvs Pharma Ltd., Mumbai, India) at the standard dose. Treatment without prior screening for parasites was chosen because of previously published evidence of submicroscopic infections in the population (15). The first two doses were given under supervision with fatty food; the remaining four doses were given to the participant/caretaker for treatment at home. All study participants received a long-lasting insecticide-treated nets (LLINs).

Data collection and follow-up

Three cross-sectional surveys were conducted; baseline (prior to treatment), after 6 weeks (post-parasite clearance) and 16 weeks (post-peak transmission season). At each survey, a single blood sample was obtained by finger prick (approximately 0·3 mL) for thick and thin blood films, filter paper blood collection (Whatman 3, Maidstone, UK), Haemoglobin test (HemoCue photometer) and for a Rapid Diagnostic Tests (RDT; Orchid Biomedical Systems, Goa, India) for malaria. Filter papers were air-dried and stored in plastic bags with silica desiccant (silica gel type III; Sigma, Dorset, UK) and stored at −20°C. Plasma was diluted 1 : 1 in 0·1% sodium azide in PBS (reaching a final concentration of 0·05%). Individuals were followed up for 6 months by passive case detection with those who experienced a clinical malaria attack (temperature >37·5°C with parasites at any density) treated according to national treatment guidelines.

Parasite detection

Parasites were detected using three methods; microscopy, RDT and PCR. For microscopy, 100 fields of a Giemsa stained thick blood film were examined during the surveys,

and at all occasions, when a clinical malaria episode was suspected, RDTs (RDT; Orchid Biomedical Systems) were used for immediate detection of infection in the field. For PCR, DNA was extracted from filter paper samples using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany), parasite detection carried out by nested-PCR amplification of the small subunit ribosomal RNA (rRNA) gene (16).

Enzyme-linked Immunosorbant Assay

Immunoglobulin G (IgG) antibodies were assayed by ELISA, as described previously (14,17). Recombinant *P. falciparum* apical membrane antigen (AMA-1 FVO, provided by Takafumi Tusboi, Ehime University, Japan), merozoite surface protein 1_{19} (MSP-1_{19} Wellcome allele, provided by Patrick Corran, London School of Hygiene & Tropical Medicine with permission of Tony Holder), merozoite surface protein 2 (MSP-2, Dd2 allele provided by David Cavanagh, Institute of Immunology and Infection Research, Edinburgh, UK), circumsporozoite protein (CSP; $NANP_{16}$ peptide, provided by Patrick Corran, London School of Hygiene & Tropical Medicine) and *Anopheles gambiae* salivary antigen (gSG6 provided by Bruno Arcà, Sapienza University, Rome, Italy) were coated onto ELISA plates overnight at 4°C at a concentration of 1.25 ug/mL for AMA1, 5 μg/mL for gSG6 and 0.5 μg/mL for all the other antigens. Plates were washed using PBS plus 0·05% Tween 20 (PBS/T) and blocked with 1% (w/v) skimmed milk powder (Marvel, UK) in PBS/T. Serum samples were added in duplicate to each plate at a serum dilution of 1 : 400 for CSP, 1 : 2000 for AMA-1, 1 : 1000 for MSP-2 and MSP-1_{19}, and 1 : 100 for gSG6 in 1% bovine serum albumin (BSA) in PBS/T. A positive control of pooled hyperimmune serum collected from adults resident in a malaria endemic area was included in duplicates on each plate in a 4-fold serial dilution from 1 : 50 to 1/51 200 (6 concentrations in total) to allow standardization of day-to-day and plate-to-plate variation.

Data analysis

All data were double entered and validated in Microsoft Access. Data were imported in STATA 12.0 (Stata Statistical Software; StataCorp, College Station, TX, USA) and the R statistical software (R Foundation for Statistical Computing, Vienna, Austria) for statistical analysis. Fever was defined as an observed axillary temperature $\geq$37·5°C and/or individual-reported fever within the previous 24 h. Patent parasite carriage as any parasite density detected by microscopy; submicroscopic parasitaemia as parasitaemia detected by PCR in the absence of microscopically confirmed parasite carriage. Parasite density was presented as geometric mean in patent parasite carriers only, together with the 25th and 75th percentiles (interquartile range, IQR).

Duplicate ELISA OD results were averaged and normalized against the positive control sample on each plate. To do this, a titration curve was fitted to the ODs obtained for the standard plasma dilutions by least squares minimisation using a three variable sigmoid model and the solver add-in in Excel 2007 (Microsoft Corp., Redmond, WA, USA), assuming an arbitrary value of 1000 U/mL of antibody against each antigen in the standard pool (5). Mean OD values for the spot extracts were converted to units/mL using this fitted curve. Sample, where duplicate optical densities (ODs) differed by more than 50%, results were excluded from the analysis. The binding of antibodies in serum from 44 Europeans never exposed to malaria was used to define a cut-off (mean OD + 3 SD) for positive and negative responses to each antigen. Antibody titre was estimated using the formula dilution/[maximum OD/(OD test serum minimum OD) − 1] where the maximum OD was the maximum value of the standard curve and the minimum OD the lowest value of the negative control. The titre expressed in Arbitrary Units (AU/mL) was used as an indicator of antibody density in the analyses. Only individuals $\geq$1 year were included in the serological analysis to minimize the effect of maternally derived antibodies in infants. Categorical variables were analysed using chi-square test or chi-square test for trend. Students *t*-test, analysis of variance or nonparametric equivalents were used when comparing continuous variables. Logistic and linear regression models were used to adjust binary and continuous variables for potential confounding. Titre values were $\log_{10}$ transformed for analyses. To assess the effect of parasite exposure on antibody titres individuals were categorized into one of the following four exposure groups: (i) parasite-free (microscopy and PCR-negative at all surveys, no clinical malaria recorded); (ii) always parasitaemic (positive at all surveys by either microscopy or PCR); (iii) lost infection (initially PCR or microscopy positive, negative at later surveys); and (iv) acquired infection (initially PCR and microscopy negative, positive at later surveys). Statistical significance of differences between these exposure groups as well as time trends within groups were determined by comparison of nested linear mixed-effects models, using likelihood-ratio tests; model fitting was performed using the LME4 package in R. All models included a random effect at the individual level to account for the within-individual correlation of titre measurements at different time points. Geographical clustering of parasite prevalence, antibody prevalence or age-adjusted antibody density was assessed as described previously (18,19) using SATSCAN software on binary (Bernouilli

model) or continuous (normal model) variables (http://www.satscan.org/, Accessed 2 February 2012).

RESULTS

Baseline characteristics and parasite carriage of study population

A total of 509 individuals were enrolled in the longitudinal study; 249 children ≤ 5 years of age, 126 children between 6 and 10 years of age and 134 adults who were ≥ 20 years (Table 1). The overall *P. falciparum* parasite prevalence by microscopy at enrolment was 38·1% (194/509). Microscopic *P. falciparum* parasite prevalence was significantly higher in children 6–10 years of age compared with younger children ($P = 0{\cdot}002$) and lowest in adults ($P < 0{\cdot}001$); parasite density in parasitaemic individuals decreased with age (test for trend between age groups, $P = 0{\cdot}012$). Baseline *P. falciparum* parasite prevalence by PCR was 57·1% (284/493) and showed the same age-pattern as microscopically detectable parasite carriage, that is, higher in children 6–10 years compared with younger children ($P < 0{\cdot}001$) and lowest in adults ($P = 0{\cdot}002$). As expected, given that all participants were given curative antimalarial therapy at enrolment, *P. falciparum* parasite prevalence decreased during the study in all age groups (Figure 1). During the last cross-sectional survey, none of the adults had microscopically detectable infections, but 14·2% (16/113) had submicroscopic *P. falciparum* infections. We found no evidence for geographical clustering of parasite carriage at any time point (data not shown).

Antibody prevalence and titre in relation to age and concurrent *P. falciparum* parasitaemia

We evaluated the prevalence and titre of antibodies against *P. falciparum* AMA-1,MSP-1_{19}, MSP-2, and CSP and against *An. gambiae* salivary protein gSG6. The baseline prevalence of antibodies to MSP-1_{19}, MSP2 and CSP all increased with increasing age group ($P < 0{\cdot}001$). Prevalence of anti-AMA-1 antibodies showed an initial increase and then decrease with age; antibody prevalence was higher in 6- to 10-year-old children compared with younger children ($P < 0{\cdot}001$) and compared with adults ($P = 0{\cdot}005$). Antibody titre increased with increasing age group for MSP-1_{19}, MSP-2 and CSP ($P \leq 0{\cdot}009$; Figure 2, Table 1). AMA-1 antibody titre was again higher in 6- to 10-year-old children compared with younger children

Table 1 Characteristics of enrolled individuals

		≤ 5 years	6–10 years	>20 years
N		249	126	134
Age	Median (IQR)	3 (2–4)	7 (6–9)	35 (27–50)
Female	Prevalence% (*n*/*N*)	50·2 (125/249)	43·7 (55/126)	64·9 (87/134)
Hb < 11 g/dL	Prevalence% (*n*/*N*)	39·0 (97/249)	15·1 (19/126)	6·7 (9/134)
Temp $\geq 37{\cdot}5$ °C	Prevalence% (*n*/*N*)	9·3 (23/247)	2·4 (3/125)	3·7 (5/134)
Protective measures				
Bed net use	Prevalence% (*n*/*N*)	73·8 (183/248)	46·3 (57/123)	66·4 (89/134)
IRS	Prevalence% (*n*/*N*)	65·5 (163/249)	61·1 (77/126)	52·2 (70/134)
P. falciparum infection				
Slide	Prevalence% (*n*/*N*)	42·2 (105/249)	58·7 (74/126)	11·2 (15/134)
	GM (IQR)	1200 (360–4360)	480 (280–1440)	200 (80–280)
PCR	Prevalence% (*n*/*N*)	54·2 (130/240)	84·4 (103/122)	37·5 (48/128)
Antibody responses				
AMA-1	Prevalence% (*n*/*N*)	64·7 (145/224)	91·7 (110/120)	78·2 (93/119)
	GM (IQR)	300 (111–853)	936 (409–1898)	413 (198–771)
MSP-1_{19}	Prevalence% (*n*/*N*)	21·2 (45/212)	28·4 (29/102)	61·5 (75/122)
	GM (IQR)	52 (25–215)	99 (32–335)	538 (135–1319)
MSP-2	Prevalence% (*n*/*N*)	42·9 (93/217)	76·1 (89/117)	81·3 (100/123)
	GM (IQR)	251 (92–649)	820 (136–3005)	1112 (360–2074)
CSP	Prevalence% (*n*/*N*)	5·9 (13/219)	31·8 (35/110)	61·2 (71/116)
	GM (IQR)	69 (34–130)	161 (84–306)	286 (144–465)
gSGS6	Prevalence% (*n*/*N*)	52·5 (125/238)	62·8 (76/121)	64·1 (75/117)
	GM (IQR)	8·5 (5·4–19·8)	11·3 (7·5–22·9)	11·9 (7·0–20·8)

IQR, interquartile range; RDT, rapid diagnostic test; GM, geometric mean.

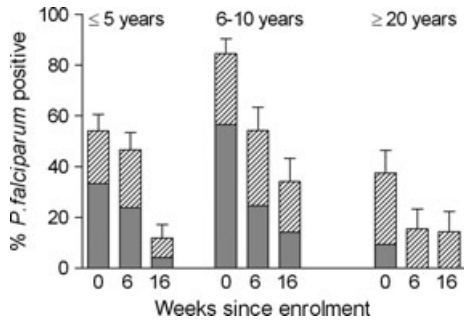

Figure 1 Parasite carriage by microscopy and PCR. Parasite carriage by microscopy (black bars) and PCR (hatched bars) for children below 5 years of age, 5–10 years of age and adults at enrolment (0 weeks), 6 and 16 weeks after enrolment. The error bar indicates the upper limit of the confidence interval around the total proportion of parasitaemic individuals.

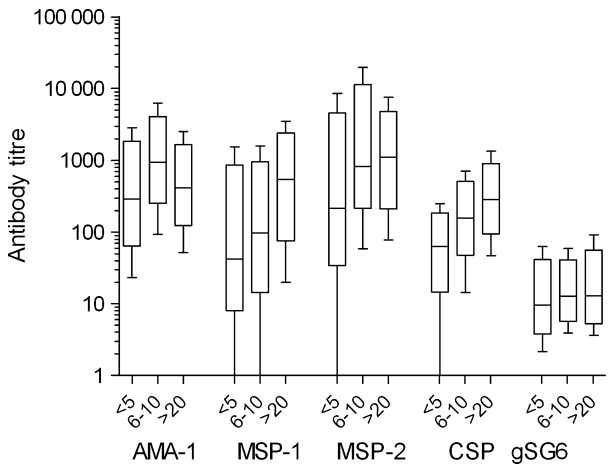

Figure 2 Antibody titres at enrolment in relation to age. Box plots indicate the median, 25th and 75th percentile antibody titre for AMA-1, MSP-1_{19}, MSP-2, CSP and gSG6 at enrolment; whiskers indicate the 5th and 95th percentile.

($P < 0{\cdot}001$) and adults ($P < 0{\cdot}001$). Baseline anti-gSG6 antibody prevalence showed a borderline significant increase with age ($P = 0{\cdot}053$); antibody titre increased significantly with age ($P = 0{\cdot}004$). We found no evidence for geographical clustering of the prevalence or age-adjusted titre of antibodies against any of the antigens at any time point (data not shown).

We determined the association between concurrent parasite carriage and antibody prevalence and titre in all age groups (Table 2). While we found no evidence for an association between parasite carriage by microscopy or PCR and concurrent antibody prevalence or titre in study participants aged 6 years and older (data not shown), parasite carriage was associated with elevated antibody prevalence and titre in younger children. When parasite carriage among 1- to 5-year-old children was categorized as parasite-free, submicroscopic infection or patent (microscopically detectable) infection, antibody prevalence increased across these categories for AMA-1 ($P < 0{\cdot}001$), MSP-1_{19} ($P = 0{\cdot}006$) and MSP-2 ($P < 0{\cdot}001$), but not CSP ($P = 0{\cdot}77$). Antibody titre increased across these categories of parasite carriage for AMA-1, MSP-1_{19}, MSP-2 and CSP (Figure 3; $P = 0{\cdot}001$). Anti-gSG6 antibody prevalence and titre also increased across these categories ($P < 0{\cdot}001$). Pairwise comparisons are presented in Table 2.

The dynamics of antibody titres in relation to the acquisition and loss of *P. falciparum* infections

We further explored the dynamics of antibody titres in relation to malaria infections in children 1–5 years of age (i) who were consistently parasite-positive throughout the study; (ii) who were parasite-free throughout the study; (iii) who were parasite-positive at enrolment but did not become re-infected after treatment; and (iv) who were parasite-free at enrolment but acquired an infection during follow-up.

Children below 5 years of age who were consistently parasite-positive during the study did not have consistently higher titres of antibodies against AMA-1 ($P = 0{\cdot}21$), MSP-1_{19} ($P = 0{\cdot}26$), MSP-2 ($P = 0{\cdot}91$), CSP ($P = 0{\cdot}29$) or gSG6 ($P = 0{\cdot}23$) compared with children who were consistently parasite-negative (Figure 4; Table 3). However, the dynamics of antibody titres were influenced by parasite exposure during the study. In children of this age group who were consistently parasite-positive, antibody titre against AMA-1 ($P = 0{\cdot}39$), MSP-1_{19} ($P = 0{\cdot}47$), MSP-2 ($P = 0{\cdot}48$) and gSG6 ($P = 0{\cdot}25$) did not change significantly with time, while antibody titres for CSP showed a statistically significant decrease ($P = 0{\cdot}011$). In contrast, we found evidence for a decline in antibody titres for AMA-1 ($P < 0{\cdot}0001$), MSP-1_{19} ($P = 0{\cdot}015$), CSP ($P = 0{\cdot}016$) and gSG6 ($P = 0{\cdot}0005$) with a borderline significant trend for MSP-2 ($P = 0{\cdot}08$) for children of this age group who were never parasite-positive by microscopy or PCR during the study. Similarly, antibody titres decreased in children who were parasite-positive at enrolment but did not become re-infected after treatment for AMA-1 ($P < 0{\cdot}0001$), MSP-1_{19} ($P = 0{\cdot}003$), MSP-2 ($P = 0{\cdot}0001$), CSP ($P < 0{\cdot}0001$) and gSG6 ($P < 0{\cdot}0001$). Children who acquired an infection during the study showed no consistent patterns in antibody titres: antibody titres for all antigens were stable or elevated 6 weeks after enrolment in children aged 1–5 years, with a decline between weeks 6 and 16 to (below) enrolment levels. When the entire period of follow-up was considered, there was a decline in titres for AMA-1 ($P < 0{\cdot}0001$), CSP ($P < 0{\cdot}0001$) and gSG6 ($P < 0{\cdot}0001$); while no statistically significant trends were observed for MSP-1_{19} ($P = 0{\cdot}79$) and MSP-2 ($P = 0{\cdot}96$) titres.

Table 2 Antibody prevalence and titre in relation to concurrent parasite carriage in children ≤5 years of age

	Parasite-free	Submicroscopic	Microscopic	*P*-value*	*P*-value**
AMA1					
Prevalence%	46·6 (183/393)	60·4 (61/101)	70·2 (94/134)	0·036	0·18
GM (IQR)	111·9 (44·4–504·2)	216·1 (82·4–994·1)	284·30 (144·0–853·0	<0·001	0·64
MSP-2					
Prevalence%	35·8 (133/372)	45·2 (47/104)	57·1 (72/127)	0·024	0·84
GM (IQR)	150·3 (68·7–583·6)	257·1 (100·8–644·5)	354·0 (24·8–160·7)	<0·001	0·23
MSP-1_{19}					
Prevalence%	14·1 (52/369)	25·3 (24/95)	20·6 (26/126)	0·047	0·16
GM (IQR)	30·6 (16·5–110·8)	51·3 (21·0–285·6)	48·8 (24·8–160·7)	0·02	0·84
CSP					
Prevalence%	3·4 (13/386)	7·7 (8/104)	3·0 (4/132)	0·29	0·032
GM (IQR)	29·2 (20·9–79·2)	41·4 (25·3–112·4)	51·1 (37·1–108·7)	0·053	0·97
gSG6					
Prevalence%	27·1 (114/421)	45·7 (53/116)	48·6 (68/140)	<0·001	0·54
GM (IQR)	5·4 (3·1–11·7)	7·0 (4·1–17·0)	8·4 (5·0–21·2)	0·14	0·12

Data from all cross-sectional surveys were combined; children therefore contribute up to three data points. Parasite-free: parasite-negative by microscopy and PCR; submicroscopic: parasite-negative by microscopy, parasite-positive by PCR; Microscopic: parasite-positive by microscopy. **P*-value for the comparison between parasite-free and children with submicroscopic infections; ***P*-value for trend-the comparison between children with submicroscopic and microscopically detectable infections. *P*-values are adjusted for multiple observations per individual.

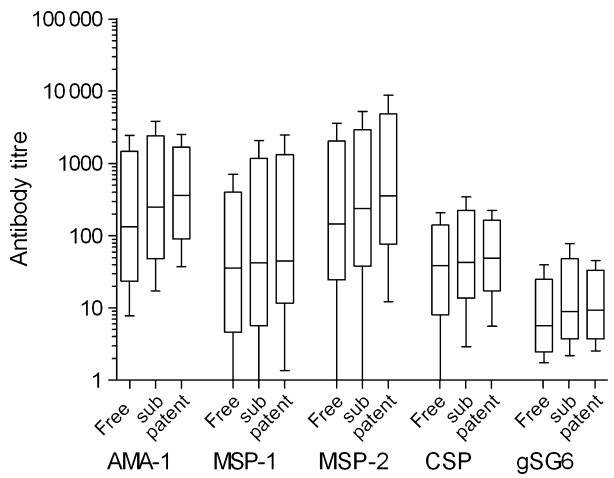

Figure 3 Antibody titres in relation to infection status in children 1–5 years old in relation to infection status. Box plots indicate the median, 25th and 75th percentile antibody titre for AMA-1, MSP-1_{19}, MSP-2, CSP and gSG6; whiskers indicate the 5th and 95th percentile. Estimates are presented for the entire study period for children aged 1–5 years old (n = 249), individual children contribute up to three observations. *Plasmodium falciparum* infection status is categorised as parasite-free (PCR and microscopy negative), submicroscopic (PCR-positive; microscopy negative) and patent (microscopy positive).

Children 6–10 years of age who were consistently parasite-positive during the study did not have significantly higher titres of antibodies against any of the antigens compared with children who were consistently parasite-negative ($P > 0{\cdot}05$ in all cases; data not shown). In children of this age group who were consistently parasite-positive, antibody titres for MSP-1_{19} ($P = 0{\cdot}41$) and CSP ($P = 0{\cdot}06$) did not change significantly with time, while antibody titres for AMA-1 ($P = 0{\cdot}002$), MSP-2 ($P = 0{\cdot}04$) and gSG6 ($P < 0{\cdot}001$) showed a statistically significant decrease over time (Table 3). We found evidence for a decline in antibody titres for MSP-1_{19} ($P = 0{\cdot}0096$), MSP-2 ($P = 0{\cdot}02$) and gSG6 ($P = 0{\cdot}0046$) but no significant differences for AMA-1 ($P = 0{\cdot}30$) or CSP ($P = 0{\cdot}055$) for children of this age group who were never parasite-positive by microscopy or PCR during the study. Similarly, antibody titres decreased in children who were parasite-positive at enrolment but did not become re-infected after treatment for AMA-1 ($P < 0{\cdot}0001$), MSP-1_{19} ($P = 0{\cdot}0002$), MSP-2 ($P < 0{\cdot}0001$), CSP ($P = 0{\cdot}0003$) and gSG6 ($P < 0{\cdot}0001$). Children who acquired an infection during the study showed no consistent patterns in antibody titres: titres declined against AMA-1 ($P = 0{\cdot}0094$), MSP-2 ($P = 0{\cdot}025$) and gSG6 ($P = 0{\cdot}021$), while no statistically significant trend was observed for MSP-1_{19} ($P = 0{\cdot}99$) and a borderline significant trend for CSP ($P = 0{\cdot}085$). In conclusion, titres declined for all antigens for children aged 6–10 years who lost their infections, but there was no consistent pattern in other groups of parasite exposure.

None of the adults were consistently parasite-positive during the study. We found evidence for a decline in antibody titres for MSP-1_{19} ($P = 0{\cdot}0023$), CSP ($P = 0{\cdot}023$) and gSG6 ($P < 0{\cdot}0001$) but no significant differences for AMA-1 ($P = 0{\cdot}22$) or MSP-2 ($P = 0{\cdot}80$) for adults who were never parasite-positive by microscopy or PCR during the study

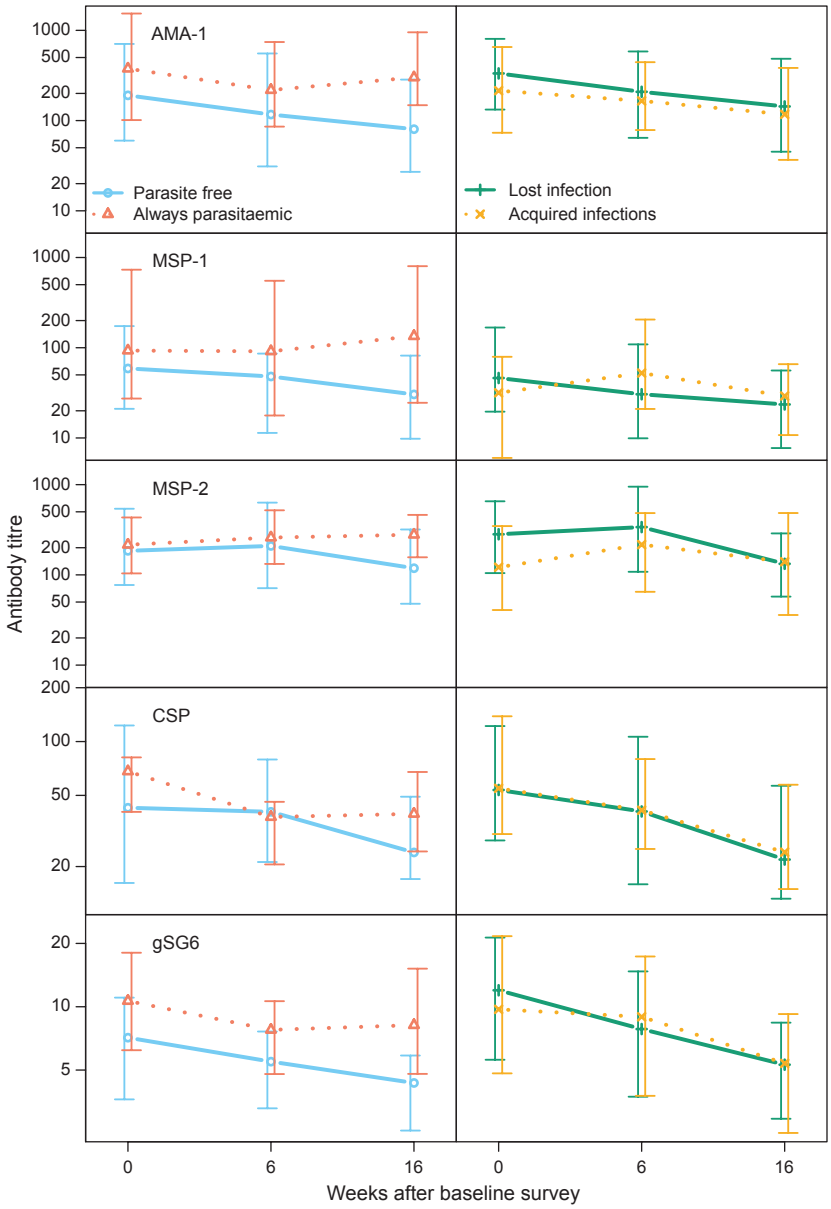

Figure 4 Dynamics of antibody responses in children 1–5 years old in relation to parasite exposure during the study. Median antibody titres for AMA-1, MSP-1_{19}, MSP-2, CSP and gSG6 during the three surveys for children who were always parasite-free (left figures, blue solid line), always parasite-positive (left figures, red dashed line), who lost infections during the study (right figures, green solid line) or who acquired infections during the study (right figures, yellow dashed line). Presented is the median antibody titre; error bars indicate the 25th and 75th percentiles.

(Table 3). We found no evidence for a change in malaria-specific antibody titres in adults who were parasite-positive at enrolment but did not become re-infected after treatment ($P > 0{\cdot}2$ in all cases), while antibody titres against gSG6 declined in this group ($P < 0{\cdot}0001$). Similarly, we found no evidence of a change in anti-malarial antibody titres for adults who acquired an infection during follow-up ($P > 0{\cdot}1$ in all cases), while antibody titres against gSG6 declined in this group ($P = 0{\cdot}0014$). In conclusion, antibody titres were mostly stable in adults with the exception of gSG6 for which titres declined during follow-up.

DISCUSSION

In this study, we describe the dynamics of malaria antibody titres in relation to microscopic and submicroscopic

Table 3 Summary of the dynamics of antibody titres in relation to age and parasite status

Age/status	AMA-1	MSP-1_{19}	MSP-2	CSP	gSG6
1–5 years					
Always parasitaemic	—	—	—	↓	—
Acquired infection	↓	—	—	↓	↓
Lost infection	↓	↓	↓	↓	↓
Always parasite-free	↓	↓	—	↓	↓
6–10 years					
Always parasitaemic	↓	—	↓	—	↓
Acquired infection	↓	—	↓	—	↓
Lost infection	↓	↓	↓	↓	↓
Always parasite-free	—	↓	↓	—	↓
>20 years					
Always parasitaemic	NA	NA	NA	NA	NA
Acquired infection	—	—	—	—	↓
Lost infection	—	—	—	—	↓
Always parasite-free	—	↓	—	↓	↓

parasite carriage in a cohort from an area of intense malaria transmission in Uganda that was cleared of their infection at enrolment.

Malaria parasite carriage has frequently been associated with higher antibody prevalence or titre in cross-sectional surveys (6,12,13,20,21). A causal association between the two is biologically plausible, that is, antibody titres being boosted by antigens in concurrent infections, because immune boosting has been observed in longitudinal studies where antibody prevalence and titre were determined before and after malaria infections (22,23), and indeed, we observed a strong association between antibody prevalence and titre for three blood-stage antigens (AMA-1, MSP-1_{19} and MSP-2) and the concurrent presence of parasite carriage at submicroscopic or microscopically detectable densities. Along with the trend in antibody prevalence and titres, being lowest in noninfected individuals, intermediate in individuals with submicroscopic parasite carriage and highest in individuals with microscopically detectable infections, this suggests that very low-density (i.e. subpatient) infections are sufficient to boost antibody titres (13). This would corroborate indications from experimental infections that very low-density infections can result in effective immune responses (24,25); although these studies both concluded that protection was most likely mediated by T cells, there was some evidence for boosting of antibody titres by low-density infections (25).

While our cross-sectional observations appear to support a role for recent infection in stimulating (or boosting) antibody titres, the apparent boosting of antibody responses against the mosquito salivary protein gSG6 indicate that the interpretation of this association is not straightforward. gSG6 antibodies indicate recent exposure to anophelines (26,27) and may be indirectly associated with malaria risk (27) but – as the proportion of mosquito bites that result in a new infection is low – there is no reason to assume that they are directly related to exposure to malaria parasites.

The association between gSG6 antibody prevalence and titre and concurrent (sub-)microscopic malaria infection illustrates the complexity of interpreting cross-sectional immunological findings. We therefore addressed the dynamics of antibody titres in relation to malaria infections in longitudinal analyses. Although longitudinal studies on malaria immunity also suffer from difficulties in distinguishing the consequences of cumulative malaria exposure (and thus accumulated immune responses to diverse antigens) from the effects of immune responses to any specific antigen (6,7), they do allow the assessment of antibody boosting and decay in the presence or absence of malaria infections. The boosting and decay of antibodies is dependent on age and cumulative exposure to malaria (28–30). We hypothesized, therefore, that antibody titres would be relatively stable in older, clinically immune individuals as all available data indicate that malaria transmission intensity has been stable (and intense) in the study area for several decades (14). We also hypothesized that in younger age groups the effect of immune boosting and antibody decay in the absence of exposure would be more pronounced (22,28).

In children aged 1–5, we found that antibody titres were not consistently higher in infected compared with noninfected children. This is likely to reflect large interindividual variation in antibody titres that are at least partly the result of variation in cumulative malaria exposure that our short longitudinal study may have failed to capture. However, while we found no statistically significant difference in antibody titres between groups of exposed and nonexposed children, we found strong evidence that the dynamics of antibody titres depend on recent parasite exposure. In children aged 1–5 years of age, we observed a decay in antibody titres during the 16-week-period of follow-up in those children who were parasite-free throughout the study or children who were not re-infected after malaria treatment at enrolment. However, a large proportion of children (56%) in this age group became re-infected within 6 weeks of drug cure and remained parasite-positive throughout follow-up, consistent with the assumptions of intense malaria transmission in this region and little para-

sitic immunity in this age group. In this group, antibody titres against all malaria antigens remained stable during the 16-week period. The vast majority of these infections were submicroscopic and restricting our analyses to these submicroscopic infections did not change this pattern of highly stable antibody titres in parasite-positive children. The fact that gSG6 antibody titres were also stable in individuals who were consistently parasite-positive but declined in children who were never parasite-positive or who were not re-infected after treatment suggests that consistently parasite-positive children were continuously exposed to anophelines. In older children (>5 years) and adults, associations between malaria infections and antibody titres were less evident. In children 6–10 years old who were parasite-positive at enrolment but did not become re-infected after clearance of their infection, antibody titres against all antigens showed a statistically significant decline. In other categories of parasite exposure, there was no consistent pattern in antibody dynamics, although antibody titres against some antigens showed a decline over time that may be a result of reduced malaria exposure during follow-up. This decreased malaria exposure during follow-up may reflect seasonal fluctuations; there is currently no clear evidence of a decline in transmission intensity as a consequence of malaria control efforts in the region (14) but indoor residual spraying was implemented with variable coverage in the region. As expected, antibody titres were largely stable in adults.

In summary, our findings suggest that, in an area of intense malaria transmission, repeated or persistent malaria infections are required to maintain antibody titres in young children but not in adults. Submicroscopic infections that are highly prevalent in all malaria endemic settings (31) appeared to provide sufficiently high levels of antigen exposure to maintain antibody titres. Our findings confirm observations in Kenyan children where antibody boosting was observed in the absence of patent malaria infections and provide evidence in support of their hypothesis that this could be explained by submicroscopic infections (32). Our data also offer support for the hypothesis that circulating antimalarial antibodies in children derive mainly from short-lived plasma cells (33) but that long-lived plasma cells may be the major source of antibodies in older individuals (34). Finally, the very rapid decline – in all age groups – in titres of antibodies to mosquito salivary gland antigens indicates that these antigens fail to induce long-lived plasma cells, suggesting that the antibodies may emanate from innate or natural B1 cells or that the antigens activate B cells in a T-cell independent manner (35).

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