Effect of the Pre-erythrocytic Candidate Malaria Vaccine RTS,S/AS01$_E$ on Blood Stage Immunity in Young Children

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(See the article by Greenhouse et al, on pages 19–26.)

Background. RTS,S/AS01$_E$ is the lead candidate malaria vaccine and confers pre-erythrocytic immunity. Vaccination may therefore impact acquired immunity to blood-stage malaria parasites after natural infection.

Methods. We measured, by enzyme-linked immunosorbent assay, antibodies to 4 *Plasmodium falciparum* merozoite antigens (AMA-1, MSP-1$_{42}$, EBA-175, and MSP-3) and by growth inhibitory activity (GIA) using 2 parasite clones (FV0 and 3D7) at 4 times on 860 children who were randomized to receive with RTS,S/AS01$_E$ or a control vaccine.

Results. Antibody concentrations to AMA-1, EBA-175, and MSP-1$_{42}$ decreased with age during the first year of life, then increased to 32 months of age. Anti–MSP-3 antibody concentrations gradually increased, and GIA gradually decreased up to 32 months. Vaccination with RTS,S/AS01$_E$ resulted in modest reductions in AMA-1, EBA-175, MSP-1$_{42}$, and MSP-3 antibody concentrations and no significant change in GIA. Increasing anti-merozoite antibody concentrations and GIA were prospectively associated with increased risk of clinical malaria.

Conclusions. Vaccination with RTS,S/AS01E reduces exposure to blood-stage parasites and, thus, reduces anti-merozoite antigen antibody concentrations. However, in this study, these antibodies were not correlates of clinical immunity to malaria. Instead, heterogeneous exposure led to confounded, positive associations between increasing antibody concentration and increasing risk of clinical malaria.

Malaria remains a global health problem [1], despite the recent increase in insecticide-treated bed-net (ITN) provision and highly effective artemisinin combination therapy [2–4]. A malaria vaccine is needed for sustained control.

RTS,S is a candidate malaria vaccine based on the circumspozoite protein (CSP) that targets the pre-erythrocytic cycle of *Plasmodium falciparum* in humans [5]. Vaccination with RTS,S has been partially efficacious against clinical malaria in the field when given with either the AS01 or AS02 adjuvant system [6, 7]. RTS,S-containing vaccines induce pre-erythrocytic immunity [8], differing from naturally acquired immunity, which largely targets blood-stage parasites [9].

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The protection conferred by RTS,S against a given sporozoite inoculum may be partial, resulting in a reduced number of merozoites being released into the bloodstream [10]. This decrease in initial merozoite inoculum may result in a quantitatively and/or qualitatively superior blood-stage immune response [11]. An alternative hypothesis is that reduced exposure to blood-stage parasites will result in reduced immunity to malaria in the long term, as described in some studies of ITN use [12].

The mediators of natural immunity to malaria are incompletely understood [9]. Nevertheless, it is known that antibodies to merozoite antigens inhibit parasite invasion of erythrocytes in vitro [13] and that their presence correlates with resistance to development of clinical malaria in prospective immuno-epidemiological studies [14].

In this study, we aimed to determine whether antibody responses to merozoite antigens are higher or lower in children receiving RTS,S vaccination, compared with control vaccinees. We therefore analyzed plasma and serum samples collected during a phase IIb randomized, controlled trial of RTS,S/AS01$_E$ among young children in Kilifi, Kenya, and Korogwe, Tanzania [7]. We assayed antibodies to 4 different merozoite antigens with use of enzyme-linked immunosorbent assay (ELISA) and assayed the growth inhibitory activity (GIA) in serum samples against in vitro parasite cultures. We analyzed the effect of vaccination on the acquisition of these serological responses and looked for correlations between these antibody responses and protection from clinical malaria episodes.

METHODS

Study Design

In Kilifi, Kenya, and Korogwe, Tanzania, 894 children aged 5–17 months were randomized in a 1:1 ratio to receive 3 doses at monthly intervals of either RTS,S/AS01$_E$ or rabies vaccine, to evaluate the efficacy and safety of RTS,S/AS01$_E$ against clinical malaria episodes by *P. falciparum* infection. Details have been published elsewhere [7]. The study protocol and its subsequent amendments received ethical and scientific approval from the Kenyan Medical Research Institute National Ethics Committee, the Tanzanian Medical Research Coordinating Committee, the Tanzania Food and Drug Authority, the Oxford Tropical Research Ethics Committee, the London School of Hygiene and Tropical Medicine Ethics Committee, and the Western Institutional Review Board in Seattle. The study was overseen by an independent data-monitoring committee and local safety monitors and was conducted in accordance with the Helsinki Declaration of 1964 (revised 1996) and Good Clinical Practice guidelines. Written informed consent in the local languages (Swahili or Giriama) was required for participation.

Monitoring for Episodes of Clinical Malaria

The primary end point was a clinical episode of malaria, defined as an axillary temperature $\geq$37.5°C, with a *P. falciparum* load $>$2500 parasites/μL. Active surveillance was implemented with weekly home visits by fieldworkers to identify febrile children. Passive surveillance was implemented by fieldworkers residing in the study villages and health care staff in local health facilities.

Blood Samples

Blood samples were taken (1) before vaccination, (2) 1 month after dose 3, (3) in March 2008 (ie, mean, 8 months; range, 4–10 months after dose 3), and (4) 12 months after dose 3. Blood samples were collected in serum separator tubes for the growth-inhibitory assay studies and into lithium heparin tubes for ELISA studies. Separated serum and plasma was aliquoted and stored at −80°C until assayed.

ELISA

Samples were tested by ELISA for the presence of human IgG against the following *P. falciparum* antigens as described elsewhere [15]: MSP-1$_{42}$, 3D7 sequence expressed in *Escherichia coli* [16]; MSP-3, FVO sequence, expressed in *E. coli* [17]; the receptor-binding domain II (PfEBA-175RII) of EBA-175, 3D7 sequence, expressed in *P. pastoris* [18]; and AMA-1, 3D7 sequence expressed in *E. coli* [19]. In brief, each antigen was coated onto high absorbance plates (Immulon4 HBX) at a concentration of 0.5 μg/mL and stored at 4°C overnight. The plates were washed 3 times in phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBS-T) and blocked for 3 h with blocking buffer (1% w/v dried skimmed milk powder in PBS-T). After 3 additional washes, 100 μL of each plasma sample were added to duplicate wells at a final dilution of 1/1000 in PBS-T. The next day, after 5 washes, 100 μL of horse radish peroxidase–conjugated antihuman IgG (DAKO) at a dilution of 1:5000 in blocking buffer was added to each well, and plates were incubated for 3 h. The plates were then developed using H_20_2 as substrate and OPD (Sigma) as the colorimetric indicator for 20 min in the dark. Plates were read at 492 nm on a Molecular Devices Versa Max ELISA reader. Tests were repeated if duplicate optical density (OD) values for an individual plasma sample varied by more than a factor of 1.5. OD readings were normalized against the 1:600 positive control dilution. A pool of serum samples from an area in Africa where malaria is highly endemic was titrated on each plate as a positive control. A 3-parameter sigmoid ligand binding model was used to least-squares fit a curve to the values of the hyperendemic serum sample pool, and this was used to calculate sample antibody concentrations on each plate.

Growth-Inhibitory Assay

Growth-inhibitory assays were performed at the Walter Reed Army Institute of Research according to previously published

methods [20, 21]. Serum samples (50 μL total volume) were dialyzed using 20K MWCO Slide-A-Lyzer Mini-Dialysis Units (Pierce) with 3 buffer exchanges against 1x PBS and a final exchange against RPMI (Invitrogen). Plasma samples were preabsorbed with human red blood cells (RBCs; blood group O) and tested for GIA by measuring the inhibition of parasite lactate dehydrogenase activity. Parasitized RBC cultures of both the 3D7 and the FVO parasite lines at the early schizont stage were established in cultures with 0.3% parasitemia and 2% hematocrit in culture medium containing 10% test plasma. Assay plates were sealed in bags containing 5% carbon dioxide, 5% oxygen, and 90% nitrogen and were incubated for 40 or 48 h (cycle time of 3D7 and FVO parasites, respectively). Cultures were then harvested by adding 80 μL/well PBS and spinning plates for 10 min at 10,000 *g*. Plates were then frozen at −30 °C until analysis. To quantify parasite lactate dehydrogenase activity, a substrate buffer containing 0.1 M Tris HCl (pH, 8.0), 50 mM sodium-L-lactate, 0.25% Triton-X, 10 mg NBT, 10 μg/mL 3-Acetylpyridine, and 10 U/mL diaphorase from *Clostridium klyiveri* (Sigma) was added to each well. Colorimetric measurement at 650 nm was done after 30 min of reaction time with use of a SpectraMax Plus 384 spectrophotometer (Molecular Devices). Calculation of GIA was determined by using the formula: percentage inhibition = [1-[(OD immune plasma—OD RBC)/(OD malaria naive plasma—OD RBC)]] × 100.

Statistical Analysis

Antibody scores from ELISAs were log-transformed to normalize distributions before analysis. Growth-inhibitory assay results were normally distributed and analyzed without prior transformation. We did not restrict fitting age and calendar date to linear effects. For instance, it is possible that, at young ages, antibody is acquired less rapidly (or even lost as maternal antibody decays). We therefore allowed for nonlinear effects by selecting multivariable fractional polynomials with use of the Royston and Altman algorithm from Stata, version 10 (StataCorp), entering age and calendar date simultaneously in the model. This allows the model to optimize the model fit using power and log functions to approximate the shape of the relationship between age and date and antibody [22]. Analyses were adjusted for fixed effects of location and bed-net use. The effect of vaccination was examined, adjusting for both the fractional polynomial fit for age and date and the antibody concentrations for each individual that was measured before vaccination. The effect of vaccination on log-transformed data was presented as a proportional difference (ie, 10 coefficient). Cox regression was used for survival analysis when the effect of antibody from all 4 times was related to clinical malaria episodes during the period of monitoring after each measurement. Therefore, each participant could contribute to 4 periods of monitoring, and the sandwich estimator was used to cluster analysis by individual [23]. Regression was also adjusted by fixed effects for age, village, distance from the health facility, and bed-net use.

Determination of Malaria Transmission Zones

SaTScan software [24] was used to calculate the spatial scan statistic [25]. The spatial scan statistic uses a scanning window that is moved across space throughout the study area. For each location and size of the window, the number of expected cases of clinical malaria is calculated, based on the expectation of an even distribution across the population. The ratio of observed to expected cases is counted, and the window with the greatest ratio of observed to expected cases is noted. We set the scan window to a maximum size of 30% of the population, where $P < .05$ and the observed to expected ratio was >2.

Distinguishing Exposure and Immunity

A logistic model was used to compare the risk of clinical malaria among all children who were definitely exposed to malaria infection. Children were therefore assigned 2 categories: cases of clinical malaria (ie, ≥1 episode of an axillary temperature >37.5°C, with a *P. falciparum* load >2500 parasites/μL) or controls (ie, asymptomatic *P. falciparum* parasitemia on either of the 2 cross-sectional surveys).

RESULTS

In total, 2915 plasma samples collected at 4 times (at screening and 1, 8, and 12 months after the third vaccination) from 866 children were tested for antibody concentrations. In addition, 2417 serum samples collected at 3 different times (at screening and 8 and 12 months after the third vaccination) were tested for growth inhibiting activity with use of 2 different parasite clones.

Age and Calendar Date

The best-fit fractional polynomials for the age profile of antibody concentrations suggested that, for AMA-1, EBA-175, and MSP-1_{42}, antibody concentrations were highest in the youngest children tested, decreased to a nadir just before 1 year of age, and then steadily increased in older children (Table 1, Figure 1a). The best fit for anti–MSP-3 antibody concentrations suggested a simple linear increase in concentrations with increasing age (Table 1). GIA steadily decreased with increasing age (Table 1).

Significant variation in antibody concentrations over time was seen for all antigens (after adjusting for age), with an initial increase, then decrease, such that the final concentrations (November 2008) were lower than the baseline concentrations (January 2007). This was not entirely synchronous with changes in malaria transmission, because antibody

Table 1. Predicted Antibody Concentrations/GIA With Age and Calendar Date, Using Results From All Four Cross-Sectional Studies

	Transformation (power)	*P*	Predicted concentration according to model. Peak/trough	Youngest age/earliest timepoint	Oldest age/latest timepoint
AMA-1					
Date	$y = m_1*x^{-2} + m_2*x^{-2}*\ln(x)$	<.0001	29.4	6.0	4.2
Age	$y = m_1*x^{-0.5} + m_2*\ln(x)$	<.0001	26.0	965.4	76.6
MSP-3					
Date	$y = m_1*x^{-2} + m_2*x^{-2}*\ln(x)$	<.0001	37.7	26.6	11.8
Age	m_1*x	.005		23.9	56.2
EBA-175					
Date	$y = m_1*x^{-2} + m_2*x^{-2}*\ln(x)$	<.0001	49.5	15.0	12.1
Age	$y = m_1*x^{-2} + m_2*x^{-2}*\ln(x)$	<.0001	29.2	473.5	63.3
$MSP\text{-}1_{42}$					
Date	$y = m_1*x^{-1} + m_1*x^{0.5}$	<.0001	26.5	14.7	8.3
Age	$y = m_1*x^{-2} + m_2*x^{-2}*\ln(x)$	<.0001	14.0	145.2	69.0
GIA for 3d7					
Date	m_1*x	<.0001	NA	29.8	16.3
Age	m_1*x	.13	NA	20.8	25.1
GIA for FV0					
Date	$y = m_1*x^{-2} + m_2*x^{-2}*\ln(x)$	.003	NA	65.8	51.9
Age	m_1*x	.065	NA	63.8	59.8

NOTE. The transformation returned by multivariable fractional polynomial is shown in the first column (x refers to antibody levels and m refers to the coefficients fit by the model). Where a linear association is reported, the *P* value given is the conventional significance of line with a gradient linear trend compared with no gradient. Where a nonlinear transformation is reported, the *P* value refers to the significance of the nonlinear transformation compared with a linear trend.

concentrations continued to decrease steadily throughout the period when the highest levels of transmission were observed during April–June 2008 (Figure 1d). GIA decreased steadily throughout the study period (Table 1, Figure 1c).

Vaccination and Antibody Concentration

Vaccination with $RTS,S/AS01_E$ was associated with statistically significant decreases in antibody concentration, with proportional differences of 0.85–0.73 for the 4 merozoite antigens tested (ie, reductions in antibody concentration were 15%–27%) (Table 2). However, the occurrence of ≥ 1 episodes of clinical malaria in the 3 months before measuring antibody concentrations had a more marked effect on antibody concentrations, with proportional differences of 2.48–10.55 (ie, antibody concentrations were 2.5- to 10-fold higher among children who had recently experienced a clinical malaria episode than among those who had not). Furthermore, adjusting the effect of vaccination for prior episodes of clinical malaria reduced both the effect size and statistical significance of the effect of vaccination (Table 2). Growth-inhibitory assay results were not influenced by vaccination but were higher after episodes of malaria.

Antibody Concentration and Subsequent Risk of Clinical Malaria

For Cox regression models, the unit of analysis was the period of observation after each antibody measurement. Therefore, each of the 866 participants could contribute up to 3 periods (after baseline and 1 and 6 months after the third vaccination). Among these time periods, there were 277 first or only episodes of clinical malaria (ie, axillary temperature $\geq 37.5^{o}C$ and parasite load ≥ 2500 parasites/μL) during 9580 months of monitoring. After adjusting for vaccination group, there were positive associations between increasing antibody concentration and an increased subsequent risk of clinical malaria for anti–AMA-1, $MSP1_{42}$, and EBA-175 antibody concentrations and for GIA (Table 3). Survival plots by antibody quartile are shown in Figure 2.

Further analysis did not provide any evidence that the subgroup of children with antibody concentrations above the median at 3 separate clinic visits (ie, over a period of 6–12 months) had lower risk of clinical malaria than did children with unstable antibody concentrations (Table 4).

Intensity of Malaria Transmission and Antibody Concentrations

To test the hypothesis that exposure to malaria infection confounds the analysis by leading to both higher antibody concentrations and ongoing higher risk of future malaria infection, we identified the geographical area with highest incidence of clinical malaria cases among the cohort with use of SaTScan. Antibody concentrations were higher in the high-transmission area (Figure 3). Antibody concentration predicted residence in the high-transmission area for AMA-1 (odds ratio [OR], 1.17; 95% confidence interval [CI], 1.06–1.29; $P = .002$), EBA-175 (OR, 1.13; 95% CI, 1.03–1.26; $P = .015$), $MSP\text{-}1_{42}$ (OR, 1.22; 95% CI, 1.12–1.34; $P < .0005$), MSP-3 (OR, 1.22; 95% CI, 1.09–1.36; $P < .0005$), GIA for 3D7

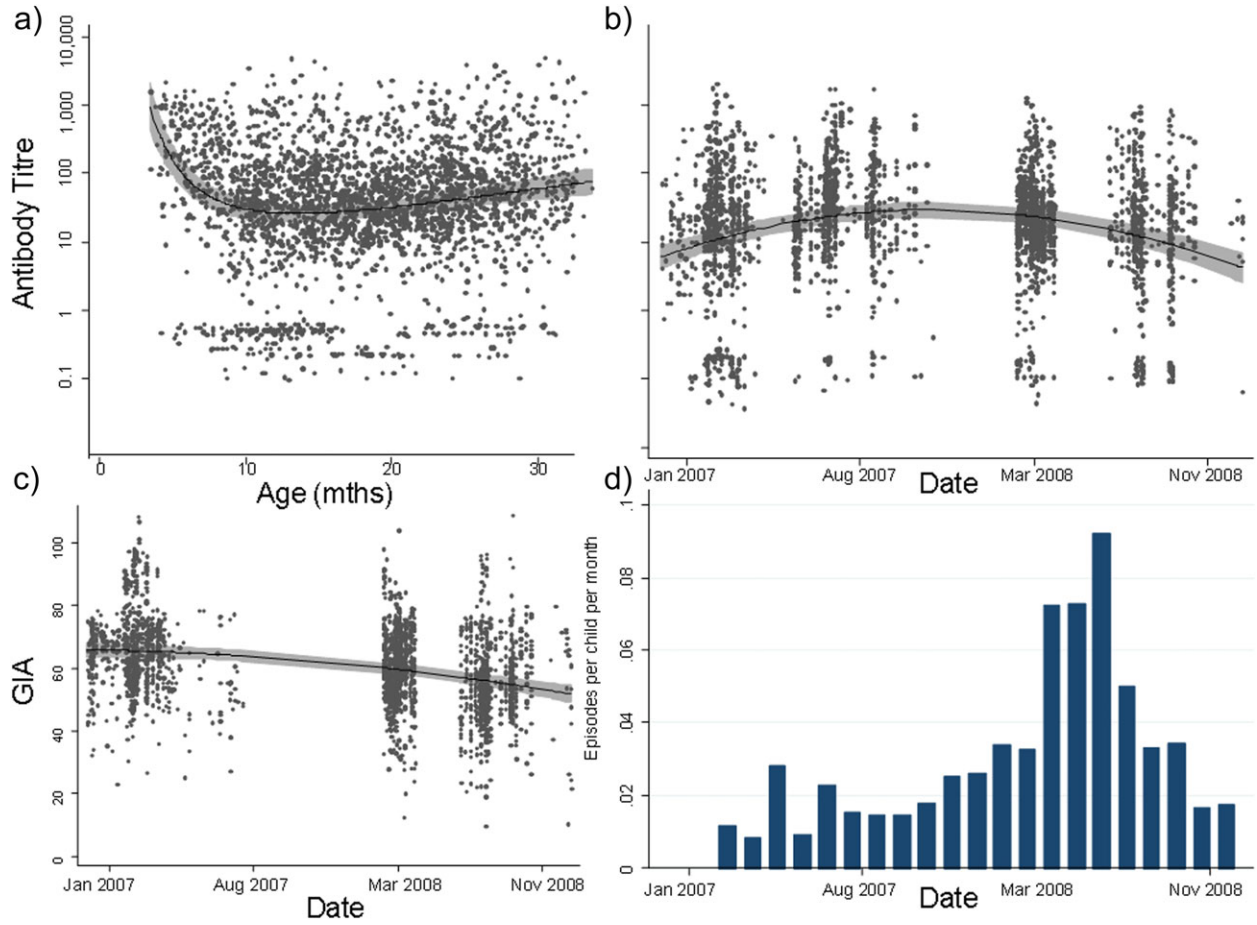

Figure 1. *A,* Scatter plot of age (x axis) against AMA-1 antibody concentration with the fitted fractional polynomial: Concentration=m_1*age$^{-0.5}$ + m_2*ln(age), where m refers to coefficients fitted by the regression model. *B,* Scatter plot of calendar date (x axis) against AMA-1 antibody concentration with the fitted fractional polynomial: Concentration=m_1*date^{-2} + m_2*date^{-2} *ln(date). *C,* Scatter plot of calendar date (x axis) against GIA for FV0 parasites with the fitted fractional polynomial: Concentration=m_1*date^{-2} + m_2*date^{-2} *ln(date). *D,* The incidence of clinical malaria per month by calendar month.

(OR, 1.13; 95% CI, 1.10–1.18; $P < .0005$), and GIA for FV0 (OR, 1.30; 95% CI, 1.24–1.37; $P < .0005$).

Distinguishing Exposure and Immunity

To reduce the confounding from variable exposure to malaria infection, we conducted a subgroup analysis of only those children who were definitely exposed to malaria infection (ie, children who had either asymptomatic parasitemia or an episode of clinical malaria) [26]. This effectively excludes the children who had no episode of clinical malaria as a result of not being exposed to infectious bites rather than as a result of being immune [27] (although the exposure could, theoretically, have

Table 2. Effect of Vaccination on Antibody Concentrations/GIA

	RTS,S/AS01$_E$ compared with control, unadjusted		Previous episodes		RTS,S/AS01$_E$ compared with control, adjusted for episodes	
	Prop dif	*P*	Prop dif	*P*	Prop dif	*P*
AMA-1	0.83 (0.7–.97)	.021	4.8 (3.65–6.31)	<.0001	0.88 (.75–1.03)	.1
EBA-176	0.85 (.73–.99)	.039	2.48 (2.03–3.05)	<.0001	0.88 (.76–1.03)	.108
MSP-142	0.73 (.59–.9)	.004	10.55 (7.41–15.02)	<.0001	0.8 (.65–.97)	.026
MSP3	0.81 (.68–.96)	.017	2.26 (1.72–2.95)	<.0001	0.84 (.71–1)	.051
GIA, 3D7 parasites	−.41% (−1.9 to +1.1%)	.591	+7.0% (3.6–10%)	<.0001	+.02% (−1.5 to +1.5%)	.98
GIA, FV0 parasites	−.8% (−2 to +.5%)	.216	+5.7% (3.4–8%)	<.0001	−.6% (−1.8 to +.7%)	.35

NOTE. Effect sizes are shown as proportional differences for log transformed antibody concentrations (ie, 2.03 = 2.03-fold increase) and absolute difference for % growth inhibitory activity.

Table 3. Cox Regression Model for Risk of Clinical Malaria Episodes (Temp ≥37.5°C and ≥2500 Parasites/μL) by Blood Stage Antigens

	All subjects		RTS,S/AS01$_E$ vaccinees		Control vaccinees	
Antigen	HR (95%CI)	*P*	HR (95%CI)	*P*	HR (95%CI)	*P*
AMA-1	1.91 (1.54–2.36)	<.0001	2.40 (1.74–3.30)	<.0001	1.68 (1.27–2.22)	<.0001
EBA-175	1.39 (1.05–1.84)	.02	1.68 (.976–2.92)	.06	1.24 (.902–1.72)	.18
MSP-1$_{42}$	1.67 (1.40–2.00)	<.0001	2.16 (1.59–2.94)	<.0001	1.48 (1.19–1.83)	<.0001
MSP-3	1.29 (1.00–1.65)	.042093	1.56 (.936–2.62)	.09	1.16 (.886–1.54)	.27
GIA for 3D7	1.15 (1.07–1.23)	<.0001	1.16 (1.05–1.29)	.003	1.14 (1.03–1.27)	.008
GIA for FV0	1.24 (1.09–1.40)	<.0001	1.20 (1.00–1.43)	.04	1.27 (1.07–1.50)	.005

NOTE. Hazard ratios (HR) and 95% confidence intervals (95%CI) are shown for each log-fold increase in antibody level. HRs are adjusted for vaccination group for the all-subjects analysis, and for village, bednet use, distance from the dispensary, period of monitoring, and age for all analyses. Age was not a significant factor (HR = 1.01, 95%CI .98–1.05).

occurred before vaccination). The analysis compared the 52 children who had asymptomatic parasitemia at either the sixth or seventh clinic visits (control participants) with the 171 children who had at least 1 episode of clinical malaria during the 3 months before these clinic visits (case participants).

The ORs for clinical malaria versus asymptomatic infection associated with increasing log-transformed antibody concentrations were not significantly different from unity: AMA-1 (OR, .87; 95% CI, 0.6–1.4; $P = .6$), MSP-1$_{42}$ (OR, .97; 95% CI, 0.7–1.4; $P = .8$), MSP-3 (OR, .87; 95% CI, 0.5–1.4; $P = .6$), EBA-175 (OR, .99; 95% CI, 0.5–1.8; $P = .9$), GIA for 3D7 (OR, 1.1; 95% CI, 0.9–1.4; $P = .2$), or GIA for FV0 (OR, 1.2; 95% CI, 0.9–1.6; $P = .17$). To take AMA-1 as an example, each 10-fold increase in antibody concentration was associated with a 13% reduction in the risk of clinical malaria as opposed to asymptomatic malaria (95% CI, –40% to 40%).

However, ITN use (OR, 2.38; 95% CI, 1.2–4.8; $P = .014$) and vaccination with RTS,S/AS01$_E$ (OR, 2.36; 95% CI, 1.1–5.0;

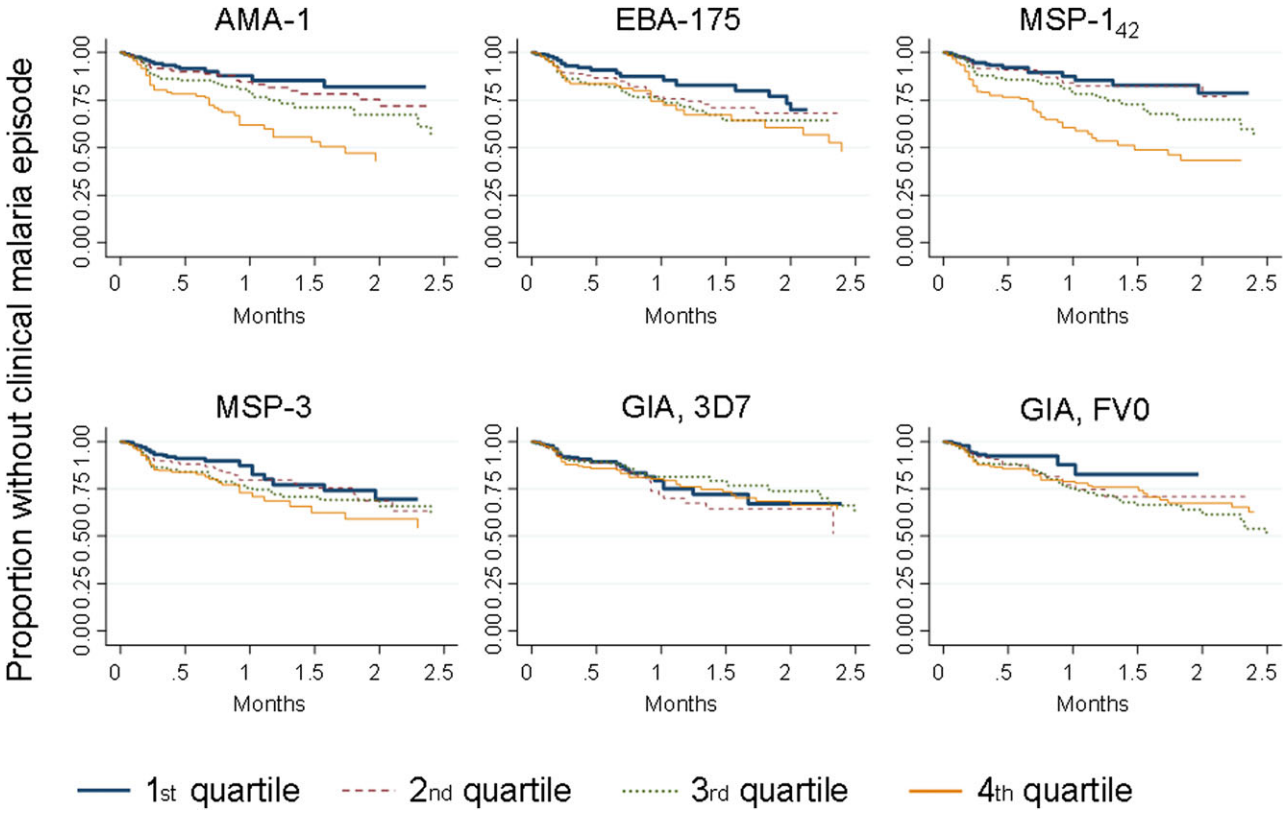

Figure 2. Kaplan Meier plots showing proportions of children without clinical malaria episodes according to quartile of antibody or GIA responses measured directly prior to periods of follow-up (ie, there are up to 3 observations for each individual: malaria incidence in the 2.5 months after antibody assessments at enrollment and at months 8 and 12 after the third vaccination).

Table 4. Cox Regression Model for Risk of Clinical Malaria Episodes (Temp ≥37.5°C and ≥2500 Parasites/μL) by Blood Stage Antigens, Classified Into Stable Low Titres, Stable High Titres, or Unstable Titres

	N	Hazard ratio	Lower CI	Upper CI	*P*
AMA-1					
Unstable	306	1			
Stable high	81	1.13	.66	1.95	.66
Stable low	65	0.40	.16	.99	.05
EBA-175					
Unstable	308	1			
Stable high	92	0.75	.43	1.31	.32
Stable low	61	0.54	.26	1.14	.11
$MSP\text{-}1_{42}$					
Unstable	276	1			
Stable high	88	1.57	.91	2.69	.11
Stable low	96	0.80	.39	1.64	.54
MSP-3					
Unstable	308	1			
Stable high	78	1.28	.69	2.38	.44
Stable low	86	1.02	.59	1.75	.94

NOTE. GIA data were not available for the 2nd clinic visit, and so could not be included. Concentrations are classified as stable and high if they are above the median for the first 3 clinic visits, and stable and low if they are below the median for the first 3 clinic visits. HRs are adjusted for vaccination group, village, bednet use, distance from the dispensary, period of monitoring, and age for all analyses.

$P = .023$) were significantly associated with increased odds of clinical malaria, compared with asymptomatic infection. Although both ITN use and vaccination reduce the risk of clinical malaria [7, 28], these interventions have an even bigger effect on reducing the risk of asymptomatic infection.

DISCUSSION

We compared antibody responses to *P. falciparum* asexual blood-stage antigens between individuals vaccinated with the malaria vaccine $RTS,S/AS01_E$ and those receiving a control vaccine. Anti-merozoite antigen antibody concentrations were lower among $RTS,S/AS01_E$–vaccinated children than among control children after adjusting for age, calendar date, and previous antibody concentration. However, the magnitude of this effect was modest in a cohort with a clinical malaria incidence of 0.53 episodes per child per year. For example, AMA-1 antibody concentrations varied by >10-fold over the age range of 5–32 months, but vaccination with $RTS,S/AS01_E$ was associated with only a 17% reduction in AMA-1 antibody concentrations. After adjusting for previous episodes of malaria, the effect of vaccination was less apparent and no longer significant, suggesting that vaccination reduces antibody levels by reducing exposure to malaria. However, previous malaria episodes could not explain all the effect of vaccination, possibly because some exposures to malaria parasites are not detected as clinical malaria cases.

Antibody concentrations initially decreased in very young children, presumably reflecting metabolic decay of maternally derived antibodies, but subsequently increased with increasing age, presumably reflecting endogenous production after malaria infection. GIA decreased with increasing age, as has been reported in previous studies [29, 30].

Among *P. falciparum*–infected individuals, ITN use and vaccination with $RTS,S/AS01_E$ were associated with increased risk of clinical malaria, compared with asymptomatic parasitemia. This suggests that the reduced risk of clinical malaria conferred by $RTS,S/AS01_E$ in this cohort [7] is partially offset by a shift from asymptomatic infection to clinical malaria. A similar shift in outcome has been described previously for ITN use [12]. $RTS,S/AS01_E$ or ITN use protects against clinical malaria and against asymptomatic infection but is somewhat more protective against asymptomatic infection than against clinical malaria. This may reflect reduced or delayed acquisition of blood-stage immunity, so that the blood-stage infections that occur in vaccinated (or ITN-using) children are more likely to progress to clinical disease. Taken together with the lower concentrations of antibodies to merozoite antigens among $RTS,S/AS01_E$ vaccinees, we conclude that the long-term protective efficacy of RTS,S vaccination is more likely to be a direct effect of the preerythrocytic immunity induced by the vaccine [31], rather than the result of enhanced acquisition of immunity to blood-stage antigens [32].

We did not clear asymptomatic parasitemia before follow-up; thus, the exposure inferred from asymptomatic parasitemia on subsequent blood samples may have occured before vaccination. However, the surveys for asymptomatic parasitemia were conducted at a mean of 8 and 12 months after vaccination, and relatively few infections persist for this long [33]. Furthermore, because vaccination group was randomly allocated, the differences in prevalence of asymptomatic parasitemia by vaccination group are likely to reflect postvaccination exposure.

Anti-merozoite antibodies and GIA were associated with highly significant increases in the prospective risk of clinical malaria. We conclude that this association was confounded by exposure to malaria infection, because (1) malaria transmission was heterogeneous in our study area, as in many other cohorts [34–37]; (2) antibody concentrations were highest in the area of highest transmission; and (3) when we analyzed the risk of clinical malaria versus asymptomatic infection (ie, excluding the uninfected children who may have been unexposed rather than immune), antibody concentrations were no longer associated with either immunity or susceptibility to clinical malaria. Malaria exposure leads to higher antimalarial antibody levels and a higher risk of clinical malaria.

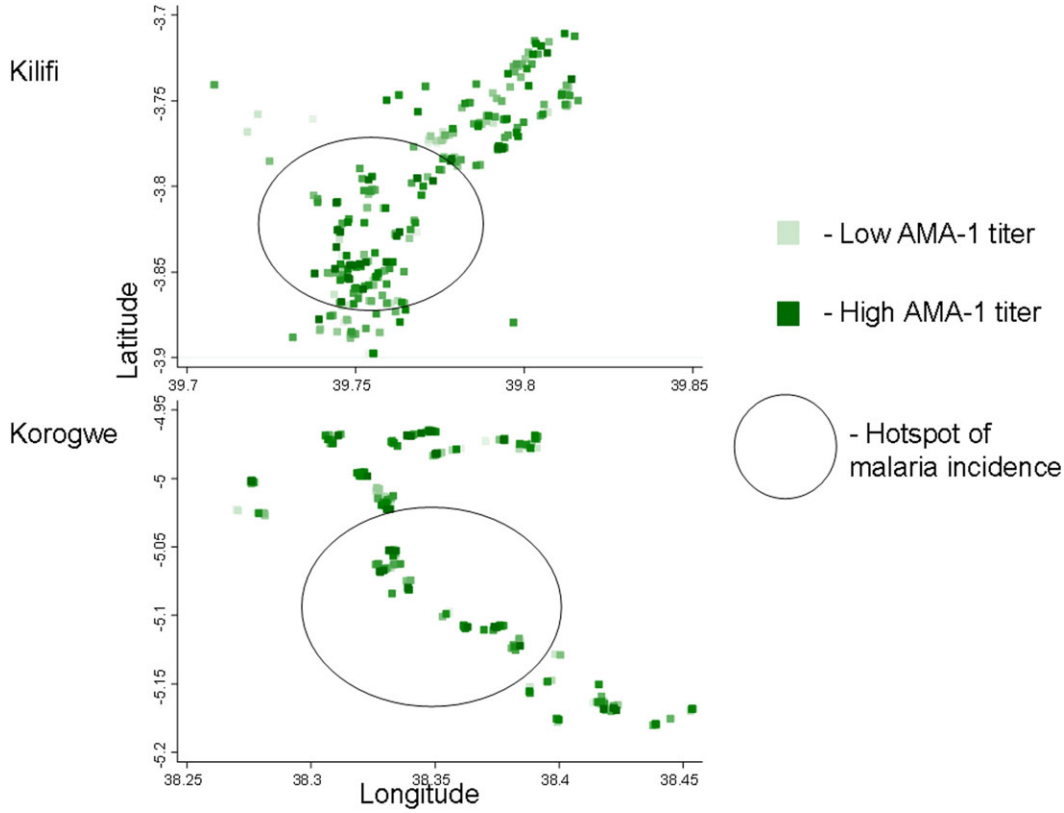

Figure 3. Maps of participants' residences, showing the relative AMA-1 antibody concentrations by intensity of shading for the green boxes, relative to the areas with highest intensity of clinical malaria episodes shown by black circles.

However, an alternative scenario might arise. As children living in high-transmission areas grow older, they will acquire immunity that protects them from clinical episodes more rapidly than in children living in low-transmission areas [38], and they will also acquire antimalarial antibodies more rapidly than will children living in low-transmission areas [37]. Therefore, in older children, there will be marked colinearity between antimalarial antibody concentrations and clinical immunity, irrespective of causality. The subgroup analyses of only the exposed children removes the confounding resulting from unexposed children at the time of sampling [26, 27] and, thus, removes the apparent increase in risk because of residence in an area of high transmission but cannot address the inevitable colinearity between antibody concentrations to different malarial antigens (including causal and noncausal associations with clinical immunity).

The lower concentrations of anti-merozoite antibodies in RTS,S-vaccinated children in our study probably reflected reduced exposure to blood-stage infections, but these antibodies were not correlates of clinical immunity. Nevertheless, independent of the anti-merozoite antibodies, when RTS,S vaccinees acquired blood-stage malaria infections, they were more likely to develop clinical disease than are unvaccinated individuals. However, the overall effect of RTS,S in protecting against all forms of malaria infection meant that RTS,S vaccinees still had a significantly lower incidence of clinical malaria than did unvaccinated individuals. Protection was sustained for at least 15 months [39].

Several unexamined caveats prevent us from concluding that antibodies to the merozoite antigens tested are not capable of mediating protection. Epitope specificity [40, 41], allele specificity [42], functional properties [43], isotype [44], avidity [45], or interactions of responses [46, 47] may be more important than concentration of antibody. If we plan to continue using immuno-epidemiological studies to examine these questions, we will need to consider testing large numbers of antigens and antibody properties simultaneously [48], adjusting for markers of exposure [26, 27, 49] and accounting for the extensive colinearity between antibody responses [50].

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