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Levels of Plasma Immunoglobulin G with Specificity against the Cysteine-Rich Interdomain Regions of a Semiconserved *Plasmodium falciparum* Erythrocyte Membrane Protein 1, VAR4, Predict Protection against Malarial Anemia and Febrile Episodes

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Antibodies to variant surface antigen have been implicated as mediators of malaria immunity in studies measuring immunoglobulin G (IgG) binding to infected erythrocytes. *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) is an important target for these antibodies, but no study has directly linked the presence of PfEMP1 antibodies in children to protection. We measured plasma IgG levels to the cysteine-rich interdomain region 1α (CIDR1α) of VAR4 (VAR4-CIDR1α), a member of a semiconserved PfEMP1 subfamily, by enzyme-linked immunosorbent assay in 561 Tanzanian individuals, who were monitored clinically for 7 months. The participants resided in Mkokola (a high-transmission village where malaria is holoendemic) or Kwamasimba (a moderate-transmission village). For comparison, plasma IgG levels to two merozoite surface protein 1 (MSP1) constructs, MSP1-19 and MSP1 block 2, and a control CIDR1 domain were measured. VAR4-CIDR1α antibodies were acquired at an earlier age in Mkokola than in Kwamasimba, but after the age of 10 years the levels were comparable in the two villages. After controlling for age and other covariates, the risk of having anemia at enrollment was reduced in VAR4-CIDR1α responders for Mkokola (adjusted odds ratio [AOR], 0.49; 95% confidence interval [CI], 0.29 to 0.88; $P = 0.016$) and Kwamasimba (AOR, 0.33; 95% CI, 0.16 to 0.68; $P = 0.003$) villages. The risk of developing malaria fever was reduced among individuals with a measurable VAR4-CIDR1α response from Mkokola village (AOR, 0.51; 95% CI, 0.29 to 0.89; $P = 0.018$) but not in Kwamasimba. Antibody levels to the MSP1 constructs and the control CIDR1α domain were not associated with morbidity protection. These data strengthen the concept of developing vaccines based on PfEMP1.

Individuals in areas where *Plasmodium falciparum* is endemic gradually develop immunity to malaria (5, 10), and vaccine development will be facilitated by gaining better knowledge about the naturally acquired immune responses that mediate protection. Antibodies which target the asexual blood-stage parasites seem to be of central importance (11, 36), and several blood-stage antigens have been implicated as targets for protection (13, 28). Of particular interest are two surface-expressed proteins: *Plasmodium falciparum* merozoite surface protein 1 (MSP1) (22) and *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (16). MSP1 is a polymorphic merozoite protein crucial for the invasion of uninfected erythrocytes. High levels of MSP1 plasma antibody against both the N-terminal and the C-terminal parts of the molecules have been associated with a reduced incidence of febrile malaria episodes in some prospective seroepidemiological studies (4, 7, 15, 32), while others did not find such an association (8, 14). PfEMP1 is a variant surface antigen (44) expressed on the surface of infected erythrocytes and plays a central role in the cytoadherence to the vascular lining. Molecules of PfEMP1 have affinity for various receptors, such as thrombospondin (33), CD36 (1), intercellular adhesion molecule 1 (2), and chondroitin sulfate A (17, 34). Sequestration of infected erythrocytes in capillaries and venules in certain tissues is a key element in the pathogenesis of severe malaria syndromes due to alterations in microcirculatory blood flow and unchecked inflammatory responses (27). Furthermore, cytoadherence allows parasites to escape clearance by the spleen (16, 21).

The family of PfEMP1 comprises a number of high-molecular-weight multidomain proteins, each composed of several Duffy-binding-like domains and cysteine-rich interdomain regions (CIDR) that can be grouped into different types (25, 40). VAR4 proteins constitute a semiconserved subfamily of large PfEMP1 molecules with complex domain structure which have been implicated in the pathogenesis of severe malaria in chil-

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dren (23), while PF08_107 encodes a group C PfEMP1 with a four-domain structure not predicted to be involved in the pathogenesis of severe malaria (19, 25). Most studies of PfEMP1 have been performed using methods such as agglutination (29) or flow cytometry (42), which detect antibodies bound to the surface of infected erythrocytes. The antibody reactivity measured in these assays is directed against variant surface antigens (VSA), and even though PfEMP1 is thought to be the major target, the exact molecular target of the antibodies measured in these assays has not been determined. The antibodies that protect pregnant women against placental malaria seem to be directed against a conserved PfEMP1 variant, VAR2CSA (37), but no study has directly linked immune reactivity to PfEMP1 and malaria protection in children. In this study, we measured plasma immunoglobulin G (IgG) levels to VAR4-CIDR1α and two MSP1 constructs by enzyme-linked immunosorbent assay (ELISA) in samples from two Tanzanian villages, characterized by marked differences in transmission intensity. The study participants were monitored for malaria for 7 months in order to relate antibody levels to VAR4-CIDR1α, PF08_107-CIDR1α, C-terminal MSP1 (MSP1-19), and an N-terminal part of MSP1 (MSP1-BL2, for MSP1 block 2) to malaria morbidity.

MATERIALS AND METHODS

Study sites and population. A longitudinal malariometric study was carried out in two villages with different malaria transmission intensities in Korogwe district in the Tanga region of Tanzania: Mkokola and Kwamasimba (approximately 15 km apart). The villages are situated at different altitudes, which in northeastern Tanzania is a proxy for malaria transmission intensity (3). Malariometric surveys were conducted, and blood samples were collected from 320 individuals, aged 0 to 59 years, from each village just before rainy season in March 2004. Informed consent was obtained from all studied individuals and/or their parents. The Ministry of Health and the Ethics Committee of the National Institute for Medical Research in Tanzania approved the study.

Hemoglobin levels were measured using a HemoCue photometer (Ångelholm, Sweden), and thick- and thin-blood smears for malarial microscopy were prepared using standard methods. Venous blood was collected into EDTA tubes and centrifuged to obtain plasma, which was frozen at −20°C. Local village helpers, so-called community-owned resource persons (CORPS), and health workers at nearby health facilities performed passive case detection during the study period. The CORPS had a training course for diagnosis and treatment of febrile episodes at the village level and were provided with first-line antimalarial drugs (sulfadoxine-pyrimethamine), paracetamol, microscope slides, blood lancets, treatment charts, febrile case detection forms, and storage boxes. The CORPS received supervision by medically qualified project staff (an assistant medical officer or clinical officer) and a laboratory technician, who conducted weekly clinics in the villages. Villagers could seek treatment at any time from the CORPS. Patients with symptoms of malaria were treated with first-line antimalarial drugs. If they had severe symptoms or did not respond adequately to the first-line treatment, they were referred to a health facility. Prior to treatment, the village helpers collected clinical information and a malaria blood smear for parasite identification. The clinical information included information about symptoms of fever and measurement of axillary temperature with a thermometer. Slides were collected on a weekly basis and read at the field research laboratory located in Korogwe Hospital by experienced research laboratory technicians. According to the village and season, the malaria diagnosis was confirmed in 50 to 70% of the patients treated by the CORPS.

Case definitions and selection of plasma samples for antibody assays. A febrile malaria episode was defined as an axillary temperature of ≥37.5°C and/or a history of fever within the previous 48 h in the presence of any asexual *P. falciparum* organisms. Anemia was defined as a hemoglobin level of <11.0 g/dl (38).

We measured antibodies to the recombinant surface antigens in 277 and 284 individuals who fulfilled the follow-up criteria from Mkokola and Kwamasimba villages, respectively. For technical reasons and constraints in the availability of plasma samples, antibodies against PF08_107-CIDR1α were only measured in 267 individuals from Mkokola. Seventy-eight out of 277 (28.2%) individuals from Mkokola (high transmission) and 48 out of 284 (16.95%) individuals from Kwamasimba (moderate transmission) had a febrile malaria episode. The age distributions of the febrile malaria cases in Mkokola were 41 out of 100 in the 0 to 4 years of age group, 17 out of 73 in the 5 to 9 years of age group, 7 out of 52 in 10 to 19 years of age group, and 13 out of 52 in the 20 to 59 years of age group. In Kwamasimba, 22 out of 113 in the 0 to 4 years of age group, 12 out of 69 in the 5 to 9 years of age group, 11 out of 50 in the 10 to 19 years of age group, and 3 out of 52 in the 20 to 59 years of age group suffered from a malaria episode. Twenty-four individuals from Mkokola and seven individuals from Kwamasimba had more than one episode of febrile malaria. For the calculation of malaria fever incidence, individuals were censored for 28 days after diagnosis before being considered at risk again.

Recombinant antigens. Recombinant proteins used in ELISA were VAR4-CIDR1α, PF08_107-CIDR1α, MSP1-19, and MSP1-BL2.

VAR4-CIDR1α was PCR amplified from genomic 3D7 DNA and cloned into the pBAD-TOPO vector (Invitrogen) using the following primers: CIDR1α-Fw, 5′-GAATTCGACAAACAAAAACAAAAATATG-3′; CIDR1α-Rv, 5′-GGGT GGTTTTCGTTGTTCACAGG-3′. For production of carboxyl-terminal V5 epitope and histidine-tagged protein, the CIDR1α insert was excised by EcoRI and PmeI digestion and then subcloned into the EcoRI and blunt-ended BglII sites of the baculovirus transfer vector pAcGP67-A (BD Biosciences). Recombinant baculovirus was generated by cotransfection of the pAcGP67-A-CIDR1α construct gene and Bsu36I-linearized Bakpak6 baculovirus DNA (BD Biosciences) into insect Sf9 cells. Recombinant CIDR1α product was expressed by infection of insect High Five cells with recombinant baculovirus. CIDR1α protein was purified from culture supernatants on CO_2^+ metal chelate agarose columns and eluted with 25 mM HEPES-KOH, pH 7.6, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 100 mM NaCl, 10% glycerol, and 100 mM imidazole.

PF08_107-CIDR1α was PCR amplified from genomic 3D7 DNA using the following primers: forward, GGGGACAAGTTTGTACAAAAAAGCAGGCT TGCGTAGTAGTAGTAGTAGT; reverse, GGGGACCACTTTGTACAAGA AAGCTGGGTCTATACCTCTGCGACGGTTTC. It was then cloned into the pDONR-201 Gateway (Invitrogen) entry vector. The insert was transferred to the pDEST-15 destination Gateway vector by site-specific recombination as described by the manufacturer. Recombinant protein was expressed as a glutathione *S*-transferase fusion in *Escherichia coli* and purified as described previously (23).

Recombinant proteins derived from C-terminal MSP1-19 and full-length block 2 sequences of culture-adapted 3D7 were expressed in *E. coli* as explained in detail elsewhere (7, 9). Control glutathione *S*-transferase protein for the antigens expressed in *E. coli* was expressed from the pGEX4T1 vector (Amersham Biosciences) and purified by affinity chromatography on glutathione Sepharose 4B (Amersham Biosciences) by previously published methods (39).

Enzyme-linked immunosorbent assay. Plasma samples (277) from Mkokola village and 284 plasma samples from Kwamasimba village were tested for the presence of IgG to PfEMP1 and MSP1 by ELISA. Briefly, wells of Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 μl of VAR4-CIDR1α (1 μg/ml), PF08_107-CIDR1α (1 μg/ml), or MSP1 (0.5 μg/ml) construct diluted in 0.1 M glycine-HCl (pH 2.75) by overnight incubation at 4°C. The plates were emptied, and any residual binding was blocked with 200 μl of PBS-BSA-Triton X-100 (1% bovine serum albumin [BSA], 0.5 M NaCl, 1% Triton X-100, in phosphate-buffered saline [PBS], pH 7.2) per well. After incubation for 1 h at room temperature, the plates were washed four times with PBS-Triton X-100 (PBS, 0.5 M NaCl, 1% Triton X-100, pH 7.4), and 100 μl of plasma and controls diluted 1:100 in PBS-BSA-Triton X-100 was added in duplicate wells and incubated for 1 h at room temperature. Following four washes in PBS-Triton X-100, plates were incubated for 1 h at room temperature with peroxidase-conjugated goat anti-human IgG (Dako, Glostrup, Denmark) diluted 1:3,000 in PBS-BSA-Triton X-100. Subsequently, plates were washed, and 100 μl of *o*-phenylenediamine substrate (0.6%; Dako) diluted in distilled H_2O with 0.05% (vol/vol) H_2O_2 was added to each well. Finally, the plates were incubated at room temperature in the dark before addition of 100 μl of 2.5 M H_2SO_4. Optical densities were measured at 492 nm (OD_{492}).

Statistical analysis. Statistical analysis of data was performed using Stata/SE version 8.2 statistical software (Stata Corporation, Tex.; http://www.stata.com). Malariometric survey and ELISA data were merged. Comparison of prevalence of malaria parasitemia and densities were done using a χ^2 test or Fisher's exact test and Spearman's rank correlation, respectively. A reference positive plasma pool was generated from plasma collected from residents of a Tanzanian village where malaria is holoendemic and a negative plasma pool from 19 Danes who have never been exposed to malaria was used in all plates. To account for day-to-day variation, antibody responses were calculated as arbitrary units (AU)

with the following formula: $[(OD_{sample\ plasma} - OD_{background})/(OD_{positive\ plasma} - OD_{background})] \times 100$. The threshold of responders was determined as the mean antibody levels of the plasma from 19 healthy Danish donors plus 2 standard deviations. These were 19.9 AU for VAR4-CIDR1α, 9.2 AU for PF08_107-CIDR1α, 2.87 AU for MSP1-19, and 1.19 AU for MSP1-BL2. Proportions of responders were compared using a χ^2 test or Fisher's exact test, and antibody responses by age group and transmission intensity strata were compared using a trend test. Spearman's rank correlation test was used to assess associations between antibody levels and age. Linear regression models were used to determine the relationship between hemoglobin or parasite density and anti-PfEMP1 or anti-MSP1-19 or anti-MSP1-BL2 IgG levels as continuous or categorical variables. Logistic regression models controlling for age, parasitemia, and use of a bed net were fitted to assess the prospective risk of febrile malaria in relation to VAR4-CIDR1α, PF08_107-CIDR1α, MSP1-19, or MSP1-BL2 IgG levels and status of response. Similarly, logistic regression models were generated for anemia, controlling for the aforementioned covariates plus sex. Since the effects of age on antibody levels and prevalence clearly differed between villages, separate models were generated for each village. For each regression model, correction for age was performed using age in years (ageyr), $(ageyr)^2$, or $\sqrt{}$(ageyr). The models presented in this paper used $\sqrt{}$(ageyr), but models using the other parameters gave similar results. Differences were considered statistically significant if P was <0.05.

RESULTS

Malariometric indices in the study village. Six-hundred forty individuals (320 from each of the two study villages) were enrolled into a prospective longitudinal malaria study in March 2004. The age-specific parasite prevalence and densities at the initiation of the study in the two study villages are shown in Fig. 1. In the lowland village, Mkokola, parasite prevalence was around 80% in children between 2 and 9 years of age, and among the 1- to 4-year-old group many children carried a high parasite load. In this village, both parasite prevalence and density dropped markedly after the age of 10 years. In the highland village, Kwamasimba, parasite prevalence peaked around 20% in the 4-year-old group and was around 10% in the adult population; most parasitemias were of low density. Moreover, in Mkokola, malaria prevalence and densities were negatively correlated with age (Spearman's rank correlation, $P < 0.001$), with children under 5 years of age carrying high burdens of malaria infection, while there was no distinct age pattern in Kwamasimba village (Spearman's rank correlation, $P = 0.397$). These findings indicate that malaria transmission in Mkokola can be characterized as intense, whereas the transmission in Kwamasimba is moderate. The difference in transmission intensity was also reflected in the malaria morbidity patterns (Fig. 2). Hemoglobin levels were markedly lower in Mkokola than in Kwamasimba in the age groups from 1 to 19 years, but among the children under 1 and in adults the levels were similar (Fig. 2A). The prevalence of anemia was very high in both villages but particularly in Mkokola, where it reached more than 80% in the 2-year-old children (Fig. 2A). In both villages, hemoglobin levels were strongly positively correlated with age (Mkokola, Spearman's rank correlation [ρ] = 0.57, $P < 0.001$; Kwamasimba, $\rho = 0.50$, $P < 0.001$). The prevalence of anemia (Fig. 2A) was higher in individuals from Mkokola than in those from Kwamasimba (Pearson $\chi^2 = 13.83$, $P < 0.001$), and anemia was negatively associated with age (Mkokola, χ^2 for trend was -6.76, $P < 0.001$; Kwamasimba, χ^2 for trend was -5.83, $P < 0.001$). Only 15 individuals, 12 from Mkokola and 3 from Kwamasimba, had hemoglobin levels of <8 g/dl. Similar malariometric indices were obtained during cross-sectional surveys conducted at the end of the 7-month follow-up (data not shown). Seventy-eight individuals developed febrile malaria episodes in Mkokola, while 48 developed febrile malaria episodes in Kwamasimba during the 7-month period of disease surveillance, and the age-specific incidence rate differed markedly between the villages: it was highest at the age of 1 in Mkokola and at the age of 4 in Kwamasimba (Fig. 2B). Thus, as expected, the difference in transmission intensity between the two villages was reflected in differences in the patterns of malaria morbidity.

The plasma levels of IgG antibodies to surface proteins of *P. falciparum* vary with age and malaria transmission intensity. The anti-VAR4-CIDR1α IgG levels were higher in residents of Mkokola (high malaria transmission) than in residents of Kwamasimba (moderate malaria transmission) (χ^2 for trend was 6.16, $P < 0.001$). In both villages there was a clear age-dependent acquisition of IgG antibodies to VAR4-CIDR1α, but antibody levels increased more rapidly and peaked earlier in Mkokola than in Kwamasimba (Fig. 3A). There was a tendency of decreasing VAR4-CIDR1α IgG levels after the age of 20 years. The proportion of responders followed a similar pattern, being higher in Mkokola than in Kwamasimba ($\chi^2 = 45.13$, $P < 0.001$), increasing with age in children but decreasing after the age of 20 years (Fig. 3A). Children in Mkokolo acquired antibodies against PF08_107-CIDR1α at an early age, and at the age of 2 more than 90% had a measurable antibody response. The mean antibody levels increased until the age of 10 years; thereafter they stabilized (data not shown).

The plasma IgG levels to MSP1-19 and MSP1-BL2 depended on donor age and the level of transmission (Fig. 3B and C). In Mkokola, the percentage of responders to MSP1-19 was high even in the young age groups, and high levels of antibodies were generally found in individuals older than 10 years. Individuals from Kwamasimba also acquired antibodies to MSP1-19, albeit at a somewhat slower pace. Antibodies to MSP1-BL2 appeared at a slower rate than antibodies to the other antigens (Fig. 3C). The results are in line with previous data on MSP1-19 (12, 14, 31) and MSP1-BL2 (7, 24).

Association between IgG antibodies and hemoglobin levels. In linear regression models controlling for age, sex, parasitemia, and use of a bed net, an increase in plasma VAR4-CIDR1α antibody levels by 100 AU was associated with an increase in hemoglobin concentration of 0.6 g/dl (95% confidence intervals [CI], 0.1 to 1.0; $P = 0.024$) in Mkokola and of 0.8 g/dl (95% CI, 0.3 to 1.4; $P = 0.005$) in Kwamasimba. There were no statistically significant associations between plasma antibody levels to PF08_107-CIDR1α, MSP1-19, or MSP1-BL2 and hemoglobin concentrations.

Logistic regression models controlling for age, sex, parasitemia, and use of a bed net were used to evaluate whether the presence of VAR4 or MSP1 antibody was associated with a reduced risk of anemia. In both villages, there was a statistically significant reduction in anemia risk among individuals with VAR4-CIDR1α antibodies (Mkokola adjusted odds ratio [AOR], 0.51; 95% CI, 0.29 to 0.88; $P = 0.016$; Kwamasimba AOR, 0.30; 95% CI, 0.15 to 0.56; $P < 0.001$). The presence of PF08_107-CIDR1α, MSP1-19, or MSP1-BL2 antibody was not statistically significantly associated with protection against anemia (Table 1).

Association between IgG antibodies to surface proteins and parasite density. To evaluate associations between antibody

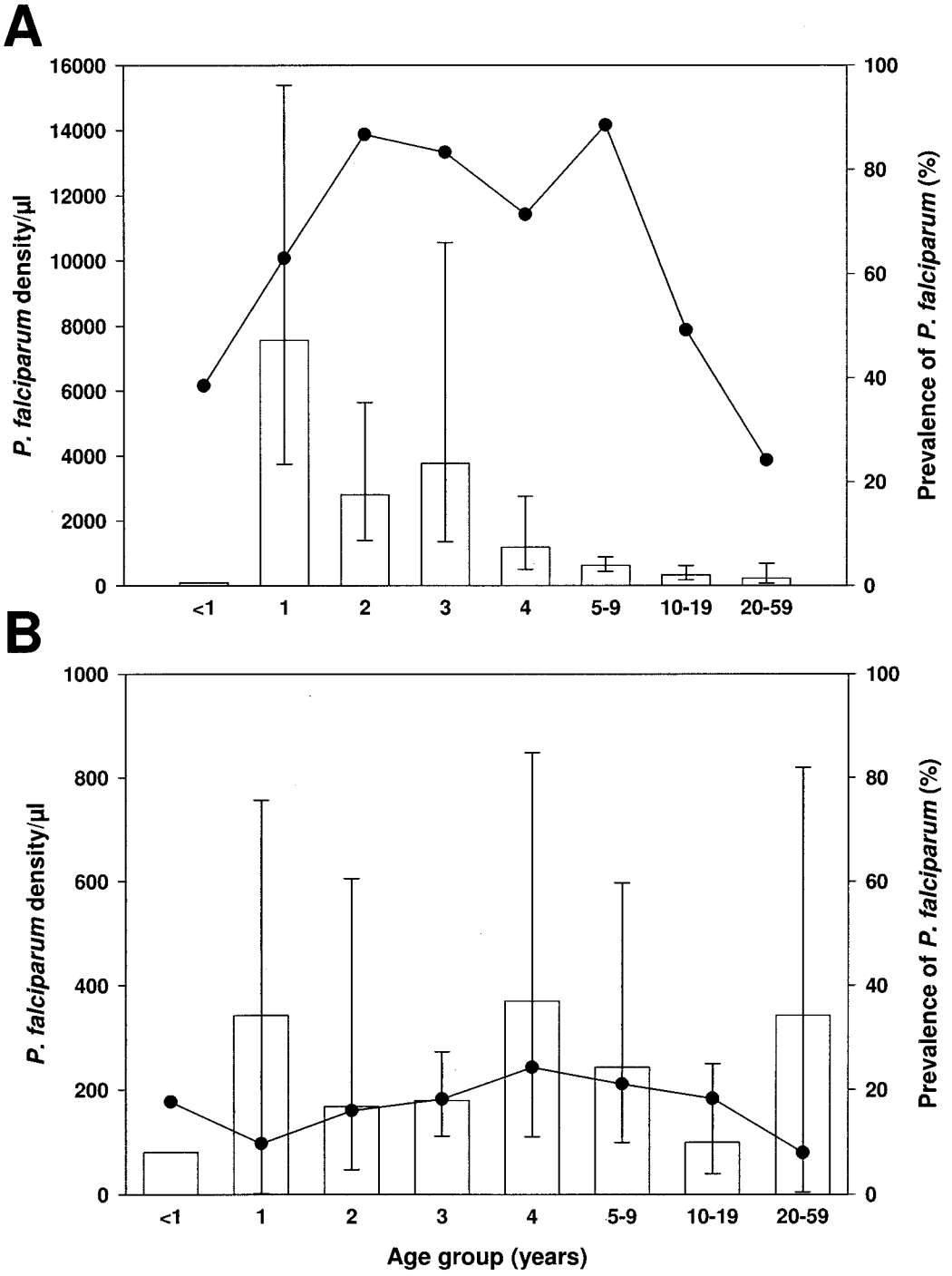

FIG. 1. Age-specific prevalence and density of *P. falciparum* in individuals aged 0 to 59 years from two Tanzanian villages situated in areas of high transmission (Mkokola [A]) and moderate transmission (Kwamasimba [B]). Error bars represent geometric means of the *P. falciparum* density with 95% confidence intervals. Note that the scale of *P. falciparum* density in panel A differs from that in panel B. Lines with filled circles represent the prevalence of *P. falciparum*.

reactivity and the ability to control *P. falciparum* multiplication, we generated linear regression models with parasite density as the dependent variable. For individuals living in Mkokola, the presence of antibodies to VAR4-CIDR1α or MSP1-BL2 was statistically significantly associated with a marked reduction of the density of *P. falciparum* parasitemia on admission (Table 2). These associations were not found in Kwamasimba, but in this village the presence of antibodies against MSP1-19 was associated with higher parasite densities (Table 2).

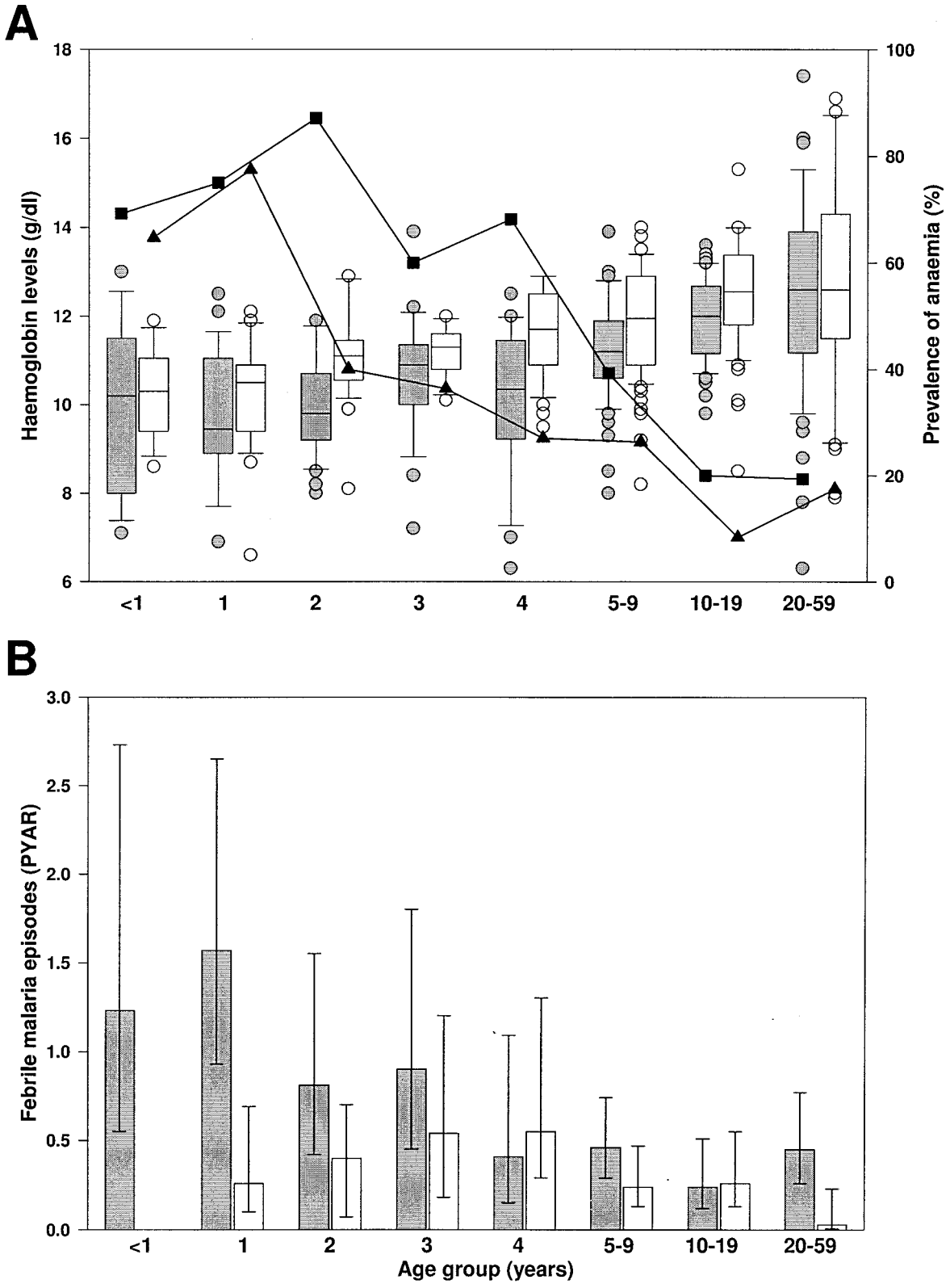

FIG. 2. Age-specific hemoglobin levels, anemia prevalence, and incidence of febrile malaria episodes in two villages characterized by different *P. falciparum* transmission intensities. (A) Hemoglobin levels and prevalence of anemia. Box plots illustrate medians with 25th and 75th percentiles and whiskers for 10th and 90th percentiles, including outliers (filled or empty circles), of hemoglobin levels. Shown are filled and empty box plots for Mkokola and Kwamasimba, respectively. Solid lines with filled squares and triangles represent the proportions with anemia for Mkokola and Kwamasimba, respectively. (B) Age-specific incidence rates and 95% confidence intervals of febrile malaria episodes per person years at risk (PYAR). Filled and empty columns represent Mkokola and Kwamasimba, respectively.

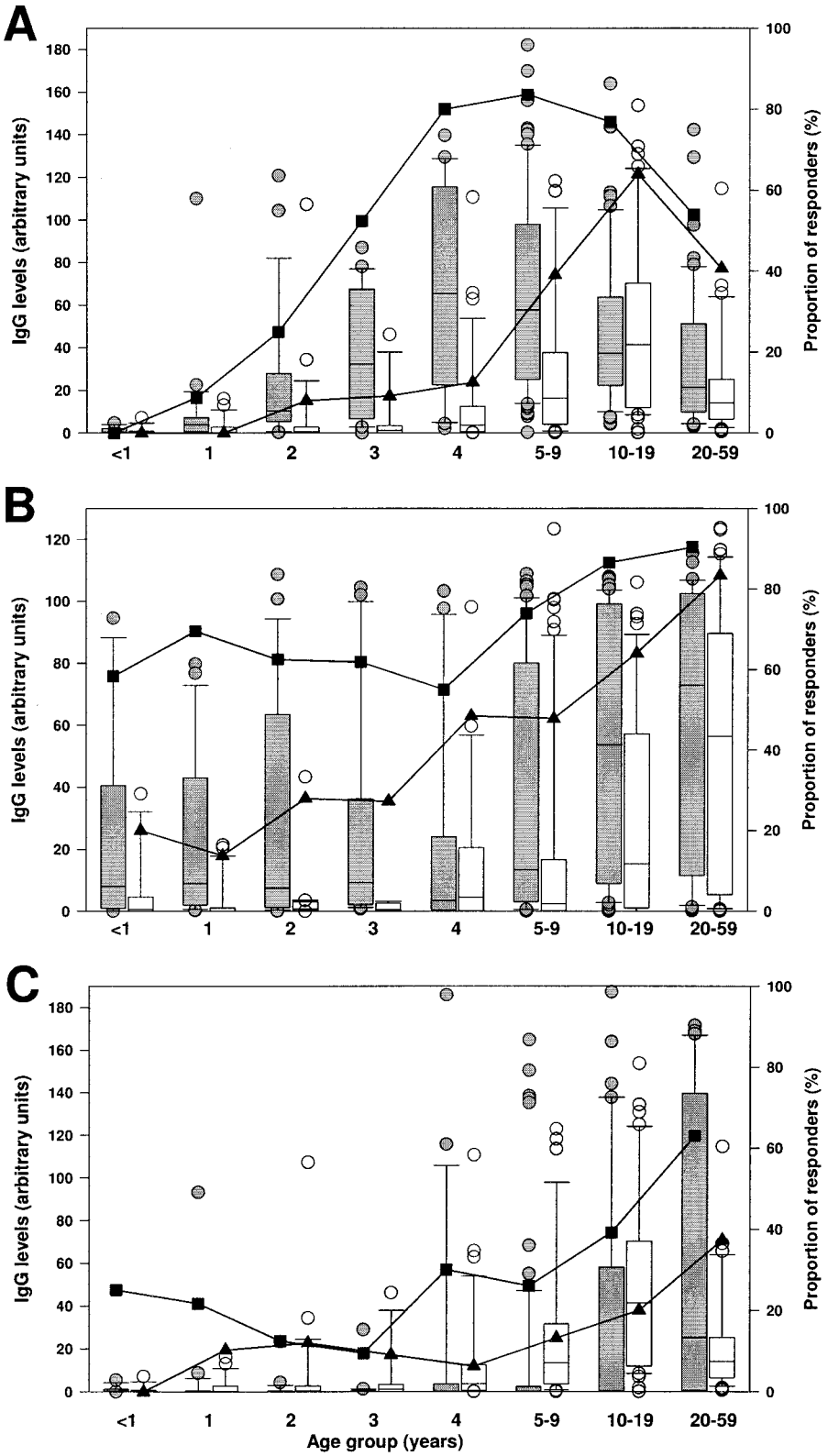

FIG. 3. Age-specific IgG responses to recombinant surface antigens in two villages characterized by different *P. falciparum* transmission intensities. Panels A, B, and C represent VAR4-CIDR1α, MSP1-19, and MSP1-BL2, respectively. Box plots illustrate medians with 25th and 75th percentiles and whiskers for 10th and 90th percentiles, including outliers (filled or empty circles), of IgG levels to each of the surface antigens. Filled and empty box plots for Mkokola and Kwamasimba, respectively, are shown. Solid lines with filled squares and triangles represent the proportion of responders to each of the surface antigens in Mkokola and Kwamasimba, respectively.

TABLE 1. Risk of anemia in individuals with antibodies to VAR4-CIDR1α, MSP1-19, or MSP1-BL2 compared to individuals without such antibodies[a]

Village	Antibody target	Unadjusted odds ratio (95% CI)	*P*	Adjusted odds ratio (95% CI)[b]	*P*
Mkokola	VAR4-CIDR1α	0.46 (0.27–0.77)	0.002	0.51 (0.29–0.88)	0.016
	PF08_107-CIDR1α	0.49 (0.22–1.10)	0.085	0.71 (0.29–1.75)	0.458
	MSP1-19	0.71 (0.41–1.23)	0.224	1.63 (0.90–2.96)	0.106
	MSP1-BL2	0.63 (0.37–1.08)	0.074	1.22 (0.64–2.31)	0.545
Kwamasimba	VAR4-CIDR1α	0.21 (0.11–0.40)	<0.001	0.30 (0.15–0.56)	<0.001
	MSP1-19	0.46 (0.27–0.77)	0.003	0.78 (0.46–1.30)	0.34
	MSP1-BL2	0.85 (0.42–1.68)	0.612	1.43 (0.64–3.19)	0.387

[a] Individuals were from a village of high malaria transmission (Mkokola) or a village of moderate malaria transmission (Kwamasimba).
[b] Adjusted for age at enrollment, sex, parasitemia, and use of bed nets.

Association between IgG antibodies to surface proteins and febrile malaria episodes. Logistic regression models correcting for age, parasitemia, and bed net use were used to compare the subsequent risk of developing a febrile malaria episode between those with and without measurable antibody responses (Table 3). The only statistically significant association was found among individuals in Mkokola, where individuals with antibodies to VAR4-CIDR1α had a relative risk of 0.49 (95% CI, 0.28 to 0.84; $P = 0.01$) of experiencing a clinical episode compared to those without such antibodies.

DISCUSSION

We have tested the hypothesis that plasma levels of VAR4-specific IgG are associated with protection from malaria morbidity. VAR4 is a high-molecular-weight PfEMP1 with a complex domain structure originally identified in 3D7, but homologues have been identified in other parasites (23). Parasites expressing VAR4 on the surface of infected erythrocytes carry a serological phenotype characteristic of parasites causing severe malaria in young children, and the molecule is considered a vaccine candidate for protecting infants and young children against severe syndromes (23). The characteristics of malaria immunity development (26) as well as the clinical epidemiology of malarial disease (20, 35, 41) are governed by the intensity, seasonality, and stability of transmission. The malaria epidemiology and morbidity patterns in the two study villages were typical for societies situated in areas of high and moderate malaria transmission. In the high-transmission village (Mkokola), infants and small children carried most of the malaria burden; they harbored high parasite densities and very often suffered from anemia, and the incidence of febrile malaria episodes peaked at the age of 1 to 2 years. In the village of moderate transmission (Kwamasimba), the burden of anemia was markedly lower than that in the high-transmission village, and the burden of febrile malaria was highest in children aged 3 to 4 years. Correspondingly, VAR4-CIDR1α antibodies were acquired at a much faster rate in Mkokola than in Kwamasimba. In both villages, the anti-VAR4 antibody levels were lower in the adults than in individuals aged 10 to 19 years, indicating that adults were less exposed to parasites expressing VAR4 than children. This is in line with earlier findings suggesting that parasites expressing PfEMP1, which mediates a very effective sequestration and confers high effective growth rates, dominate infections in nonimmune individuals (6, 30). According to this paradigm, the acquisition of antibodies against the most effective PfEMP1 binders inhibits the growth of parasites expressing these molecules, leaving the stage to parasites expressing PfEMP1 that mediate less effective binding and have a lower effective growth rate. Thus, it would be predicted that immune adults would be less exposed to VAR4-expressing parasites than children (23). By contrast to the VAR4 antibody levels, the antibody levels to the two merozoite antigens were higher in adults than in children.

In both villages, low adjusted odds ratios for anemia were found among individuals with measurable VAR4 plasma antibodies. The antibody levels to variable surface antigens of chondroitin sulfate A-selected parasite lines are positively associated with hemoglobin levels in pregnant women (43), but to our knowledge, this is the first report linking malaria antibody reactivity against a blood-stage antigen to protection against anemia among children.

TABLE 2. Relative *P. falciparum* density in individuals with antibodies to VAR4-CIDR1α, MSP1-19, or MSP1-BL2 compared to individuals without such antibodies[a]

Village	Antibody target	Unadjusted regression coefficient (β) (95% CI)	*P*	Adjusted regression coefficient (β)[b] (95% CI)	*P*
Mkokola	VAR4-CIDR1α	−3,968 (−6,999, −937)	0.01	−3,752 (−6,772, −732)	0.015
	PF08_107-CIDR1α	−1,186 (−593, 3,120)	0.588	69 (−6,113, 6,252)	0.982
	MSP1-19	190 (−2,193, 2,574)	0.875	1,397 (−1,321, 4,114)	0.313
	MSP1-BL2	−3,876 (−5,810, −1,942)	<0.001	−2,575 (−4,153, −996)	0.001
Kwamasimba	VAR4-CIDR1α	69 (−212, 349)	0.631	−75 (−522, 372)	0.741
	MSP1-19	271 (26, 516)	0.03	293 (44, 542)	0.02
	MSP1-BL2	311 (−162, 785)	0.197	211 (−373, 794)	0.478

[a] The volunteers were from a village characterized by high malaria transmission (Mkokola) or a village with moderate malaria transmission (Kwamasimba).
[b] Adjusted for age at enrollment and use of bed nets.

TABLE 3. Risk of febrile malaria in individuals with plasma antibodies to VAR4-CIDR1α, MSP1-19, or MSP1-BL2 compared to individuals without such antibodies[a]

Village	Antibody target	Responder status[b] (*n*)	No. of events[c]	PYAR[d]	Incidence (episodes/PYAR) (95% CI)	Adjusted odds ratio (95% CI)[e]	*P*
Mkokola	VAR4-CIDR1α	0 (113)	43	53.7	0.80 (0.59–1.08)		
		1 (164)	35	84.7	0.41 (0.30–0.58)	0.49 (0.28–0.84)	0.01
	MSP1-19	0 (69)	26	31.3	0.83 (0.56–1.22)		
		1 (208)	52	107	0.49 (0.37–0.64)	0.67 (0.36–1.23)	0.193
	MSP1-BL2	0 (186)	54	89.6	0.60 (0.46–0.79)		
		1 (91)	24	48.8	0.49 (0.33–0.73)	1.13 (0.60–2.13)	0.714
Kwamasimba	VAR4-CIDR1α	0 (196)	35	104.1	0.34 (0.24–0.47)		
		1 (88)	13	48.6	0.27 (0.16–0.46)	0.66 (0.28–1.58)	0.356
	MSP1-19	0 (143)	24	77	0.31 (0.21–0.47)		
		1 (141)	24	76.8	0.31 (0.21–0.47)	1.21 (0.51–2.86)	0.545
	MSP1-BL2	0 (236)	39	126.4	0.31 (0.23–0.42)		
		1 (48)	9	25.1	0.36 (0.19–0.69)	1.39 (0.59–7.30)	0.449

[a] The volunteers were from a village characterized by high malaria transmission (Mkokola) or a village with moderate malaria transmission (Kwamasimba).
[b] Status of response to recombinant antigen as defined by the cut-off (0, nonresponder; 1, responder).
[c] Number of multiple febrile malaria episodes.
[d] PYAR, person years at risk.
[e] Adjusted odds ratios were calculated in logistic regression models by comparison of individuals having or not having febrile malaria episodes during follow-up between responders and nonresponders to VAR4-CIDR1α, MSP1-19, and MSP1-BL2. The models were adjusted for age at enrollment, parasitemia, and use of bed nets.

In Mkokola, children who were responders to VAR4 antibodies also carried substantially lower parasite densities than children who were nonresponders during the initial cross-sectional survey, and although a similar association was not found in Kwamasimba, probably because parasite densities generally were low, the data indicate that children with VAR4 antibodies were more effective in controlling parasite multiplication and thus less likely to develop anemia. In immunoepidemiological studies it is not possible to distinguish whether association between clinical outcome and an immune reactivity is causal or due to covariation with other protective mechanisms. Thus, even though the antibodies were directed against VAR4-CIDR1α, the data do not exclusively prove that they were induced by VAR4 in vivo, and previous data have indicated that some CIDR domains do share cross-reactive epitopes (18); however, this does not seem to be the case for VAR4-CIDR1α (unpublished results). Individuals with VAR4-CIDR1α antibodies at the initiation of the study also had a significantly reduced risk of getting febrile malaria during the follow-up period. This was unexpected and probably due to covariation, since VAR4 antibodies would be predicted to protect against parasites expressing phenotypes linked to severe malaria but not necessarily against parasites expressing phenotypes that are less virulent but still capable of causing uncomplicated febrile disease.

This study was not designed to and did not detect association between MSP1 antibody levels and clinical protection. Associations between MSP1-19 antibody levels and protection from febrile malaria have been reported in some studies (15, 32) but not confirmed in others (14). The reason for this discrepancy is probably that the assay employing recombinant MSP1-19 constructs detects all anti-MSP1-19 antibodies which can either inhibit merozoite invasion (inhibitory antibodies) or block the inhibitory antibodies, and the fine specificity of these antibodies is crucial to the biological effects (12, 31). In the present study, no attempt was made to quantify MSP1-19 antibodies with blocking or inhibitory effects. Previous association between MSP1-BL2 antibodies and protection from malaria fever was mainly found among the IgG3 subclass (7), which was not measured specifically in this study. Interestingly, however, it is worth noting that the presence of MSP1-BL2 antibodies in villagers in Mkokola was strongly associated with marked reductions in circulating parasite densities. These anti-merozoite antibodies may well play a role in controlling parasitemia levels.

In conclusion, this study demonstrates that antibodies directed against CIDR1α of a member of the semiconserved PfEMP1 family, VAR4, were acquired early in life by children living in areas where malaria is endemic. The presence of the antibodies was associated with a marked reduction in anemia risk and, in the area of intense transmission, also with a reduction in parasite densities and incidence of malaria fevers. The data strengthen the concept of developing vaccines based on PfEMP1 to protect infants and young children in areas where malaria is endemic from severe malaria.

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