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Plasmodium falciparum Genotypes, Low Complexity of Infection, and Resistance to Subsequent Malaria in Participants in the Asembo Bay Cohort Project†

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To assess the relationship between the within-host diversity of malaria infections and the susceptibility of the host to subsequent infection, we genotyped 60 children's successive infections from birth through 3 years of life. MSP-1 Block2 genotypes were used to estimate the complexity of infection (COI). Malaria transmission and age were positively associated with the number of K1 and Mad20 alleles detected (COI_{KM}) ($P < 0.003$). Controlling for previous parasitemia, transmission, drug treatment, parasite density, sickle cell, and age, COI_{KM} was negatively correlated with resistance to parasitemia of >500/μl ($P < 0.0001$). Parasitemias with the RO-genotype were more resistant than those without this genotype ($P < 0.0000$). The resistance in low COI_{KM} infections was not genotype specific. We discuss the impact of genotype-transcending immunity to conserved antigenic determinants. We also propose a diversity-driven immunomodulation hypothesis that may explain the delayed development of natural immunity in the first few years of life and suggest that interventions that decrease the COI_{KM} could facilitate the development of protective immunity.

Children born in malaria-holoendemic areas are infected almost constantly, but it takes 3 to 5 years to develop immunity that confers protection against parasitemia and illness. The many allelic forms of asexual blood-stage *Plasmodium falciparum* antigens might contribute to this delayed acquisition of immunity. Children are infected with different parasite genotypes, bearing different allelic forms of antigens, over successive infections and within a given infection. An infection can have multiple different genotypes due to superinfection and mosquitoes inoculating multiple genotypes during a single bite. The extent of multiple-genotype infections sheds light on malaria transmission, parasite diversity, and the development of immunity.

The Block2 domain of merozoite surface protein 1 (MSP-1) and other highly diverse single-copy genes has been used to estimate the minimum number of different parasite genotypes present within *P. falciparum* infections (9). This estimate has been referred to as the complexity of infection (COI) (37). The sequence differences and tandem repeat polymorphism result in fine characterization of Block2-defined parasite genotypes. MSP-1 Block2 appears to be under diversifying selection (36 and references therein), and the linkage disequilibrium found in *P. falciparum* precludes MSP-1 Block2 genotyping from being considered a marker for other genes or loci (13, 39). We used MSP-1 Block2 genotyping in this study for two main reasons: (i) the high level of MSP-1 Block2 diversity makes it a better estimator of COI than less polymorphic "neutral" parasite genotyping loci (such as a single microsatellite loci) and (ii) MSP-1 is a candidate for having a direct relationship with the acquisition of immunity (7, 8, 14, 17).

In addition to indicating host susceptibility to malaria transmission, the COI might impact the development of immunity in either a positive or a negative way. Infection with different genotypes might lead to the development of genotype-specific or allele-specific immunity. If this occurs, a host would only develop resistance to an immunologically defined genotype, remaining susceptible to others (22). This theory predicts that multiple-genotype infections would lead to more rapid development of anti-malaria parasite immunity, since the immune response is exposed to a greater amount of the allelic diversity in one infection (22, 23). However, several studies point towards selection for coinfection under natural conditions (4, 33). Multiple-genotype infections would assist continued transmission in an area of high and year-round (holoendemic) malaria if genotypes "cooperated" to delay the acquisition of immunity by causing immunologic antagonism, presenting a "smoke screen" of antigens that result in immunomodulation and/or distracting or interfering with responses to protection-inducing antigens (2, 21, 34, 40). In addition to the many different antigens presented within a single-genotype infection, these immunologic phenomena can be extended to consider the allelic diversity presented in multiple-genotype infections, especially the high level of antigenic allelic diversity resulting from polymorphic repetitive antigens such as MSP-1 Block2.

Molecular epidemiologic studies have considered the relationship between the MSP-1-based COI and disease severity but have yielded conflicting results (1, 2, 6, 12, 16, 18, 19, 20, 30, 37, 43). The definition of disease severity and sampling time might explain the discrepancy. If sampling is done late in infection, depending on the immune status of the host and/or infection-induced immunosuppression, only fast-growing par-

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asite genotypes might be detected. It is also possible that high fever, a defining characteristic of clinical malaria, might clear parasite genotypes from an individual. The fact that in addition to these confounding variables, the COI in an individual can be affected by the immune status or age of the host, previous treatment history, malaria transmission, and parasite density necessitates studying the susceptibility to subsequent parasitemia.

Previous longitudinal studies have lacked the sample size and follow-up necessary to investigate successive infection (1, 12, 16, 19, 27, 38, 43). In the few children (mostly >4 years old) followed for up to 1 year, successive infections contained different parasite genotypes. This alternation in genotypes could be attributed to allele-specific immunity or subsequent infections containing different genotypes by chance alone. Some studies report that a higher COI was associated with increased risk to subsequent clinical malaria (1). Others report that a high COI was associated with protection against infection (12).

In this study, we investigated the parasite genotypes in western Kenyan children followed prospectively biweekly from birth to approximately 3 years. Detailed information on entomologic, clinical, and host genetic aspects of the study participants allowed a comprehensive analysis of the COI and alternating genotypes found in successive parasitemias. We elucidated factors related to children's ability to limit parasitemia and clear parasite genotypes and found that high within-host antigenic diversity is associated with impaired subsequent resistance to parasitemia.

MATERIALS AND METHODS

Study design. As part of the prospective, longitudinal Asembo Bay Cohort Project (ABCP), pregnant mothers were identified and, after consent, enrolled in the study along with their delivered infants. Children were visited at their house every 2 weeks for clinical history and contributed routine monthly blood samples for approximately 3 years. The parasite density (per microliter of blood) and species present were determined by microscopy. The children's axillary temperature and hemoglobin density were measured. During the period of this study, Fansidar (sulfadoxine-primethamine) treatment was administered if the child had a fever of ≥37.5°C with any density parasitemia or a parasite density of >10,000/μl. Sickle cell hemoglobin genotyping was also performed.

Malaria transmission. Mosquitoes were caught and tested for *P. falciparum* sporozoites by enzyme-linked immunosorbent assay (ELISA). The sporozoite seropositive rate was averaged by village by week and multiplied by the number of fed mosquitoes found in the households' bednet trap each night studied. The entomologic inoculation rate (EIR) was then averaged by village by week. We considered long-term, geographic, temporal, and seasonal EIR differences. For analysis of COI, we defined the hyper- and high-transmission seasons as lagging 1 month after the dates separating the transmission seasons. For the time resistance analysis, time periods occurring 1 month before the beginning and 2 months before the end of the hypertransmission season received greater exposure during the following 2 months. We termed this time period as hyperexposure period and the other time periods as high-exposure periods.

Genotyping. We determined the MSP-1 Block2 alleles present in 686 blood samples from children *P. falciparum* parasitemic by microscopy. A total of 1,121 parasitemias were considered in the study; however, we only genotyped blood samples with parasitemia and ≥200 μl of packed erythrocytes (RBCs) available. Also, we excluded the two samples following 1 and 3 days after a parasitemia treated with Fansidar. The DNA in the approximately 300/μl packed RBCs was extracted using the PureGene extraction method (Gentra Systems). We used a nested PCR method. The first, external PCR (55°C annealing, 25 cycles) was done with 5′-AAGCTTTAGAAGATGCAGTATTGAC and 3′-ATTCATTAA TTTCTTCATATCCATC primers. The second PCR (64°C annealing, 30 cycles) entailed (i) K1 5′-GAAATTACTACAAAAGGTGCAAGTC and 3′-AGATGA AGTATTTGAACGAGGTAAAGTG, (ii) MAD20 5′-GCTGTTACAACTAG TACACC and 3′-TGAATTATCTGAAGGATTTGTACGTCTTGA, or (iii)RO 5′-GCAAATACTCAAGTTGTTGCAAAGC and 3′-AGGATTTGCAGCACC TGGAGATCT. For the PCR mixture, 5 μl of the 5′ and 3′ primers (40 ng/μl) were added to 16 μl of deoxynucleoside triphosphate (dNTP) mixture (Promega, 80 μmol/reaction), 3 μl of $MgCl_2$ (25 mM), 10 μl of PCR 10 × buffer containing 15 mM $MgCl_2$ (Perkin Elmer), 0.5 μl of *Taq* polymerase, and 59.5 μl of double-distilled water. For the external PCR, 5 μl of DNA (adjusted to 150 ng/μl) was added to 95 μl of reaction mixture. For each internal PCR, we used 5 μl of external PCR product.

Length differences in the alleles were determined using 3% agarose–1000 gel electrophoresis, achieving 10-bp resolution with long running times and a 25-bp ladder (Gibco-BRL). Alleles were characterized by their main family sequence (K1, Mad20, and RO) and length. Positive and negative controls were included for each main family on each gel.

We confirmed the sensitivity and accuracy by repeating our PCR method on 30 samples three times throughout the study. To make the test stringent, we selected samples with high COIs (mean and standard error [SE] 5.2 and 0.37). Of the 162 alleles detected in the samples in the first reaction, the same allele was detected in the second reaction 94% of the time. The number of alleles detected in the two reactions differed by an average of only 0.05 (SE 0.01). Of the 686 samples genotyped, 99% contained at least one MSP-1 Block2 allele. Five of the 10 negative-by-PCR samples had parasite densities of ≤640/μl.

Statistical analysis. Statistical analysis was limited and hypothesis driven to avoid spurious significant results. Proportions were tested using chi-square analyses. We considered factors associated with the COI_{KM} and the ability to remain resistant for >2 months. Upon finding significant associations, we considered the mean time of resistance in months. On average, three blood samples were collected in the 2 months after a given parasitemia. These analyses were performed using a general linear model (GLM), controlling for repeated measures.

To determine estimated effects and relative risks, we used the Statistical Analysis Software (SAS, Cary, N.C.), program's procedure termed genmod. Briefly, this analysis calculates the estimated effect (EE) after considering the EE of the other variables. The different levels of a variable (for instance, high, medium, and low EIR are three different levels) are compared to a baseline. Customarily, the variable level with the lowest value is chosen as the reference (baseline) (for instance, the village with the lowest expected COI could be used as the reference for comparing the COI in different villages). However, the results are not impacted by which variable level is used as the reference. When considering a binomial outcome, the result can be translated into a relative risk, where the reference has a relative risk of 1. The results reflect the estimated effect attributable to a given variable after considering the impact of all the variables in the analysis. Nonoverlapping 95% confidence intervals (CI) can be used as a conservative test of differences among variable levels.

The expected probability of redetection of an allele in the subsequent 2 months was compared to the observed using a chi-square test for each K1 allele separately. We did not perform this test on the Mad20 alleles or RO alleles, since they were redetected in most instances. The observed frequency of redetection in blood samples in the subsequent 2 months was determined as follows. On average, we collected three blood samples between the parasitemia of interest and 2 months later; therefore, the expected probability was $N(1 - p)^3$, where N is the times the allele was detected and P is the frequency the allele was detected.

RESULTS

Parasite diversity. Our working hypothesis was that the COI, as measured by Block2 genotying, affects resistance to subsequent parasitemia. For reasons explained below, the lack of MSP-1 Block2 repeats in parasites with the monomorphic Block2 RO allele (18) was hypothesized to result in a different development of resistance to subsequent parasitemia than parasites having the repeat-containing Mad20 and K1 Block2 alleles. Therefore, we estimated the COI by the K1 and Mad20 alleles only (COI_{KM}) and then classified by RO presence.

The frequency of detecting each allele in the 668 parasitemias genotyped was determined (Fig. 1). By microscopy, 92% of the parasitemias detected contained only *P. falciparum* parasites. *P. ovale* and *P. malariae* were detected in 4 and 3% of the parasitemia, respectively. Less than 2% of *P. ovale* and *P. malariae* were pure infections.

COI_{KM}. Ninety-three percent of the parasitemias had one or more K1 allele, 79% had one or more Mad20 allele, and 82%

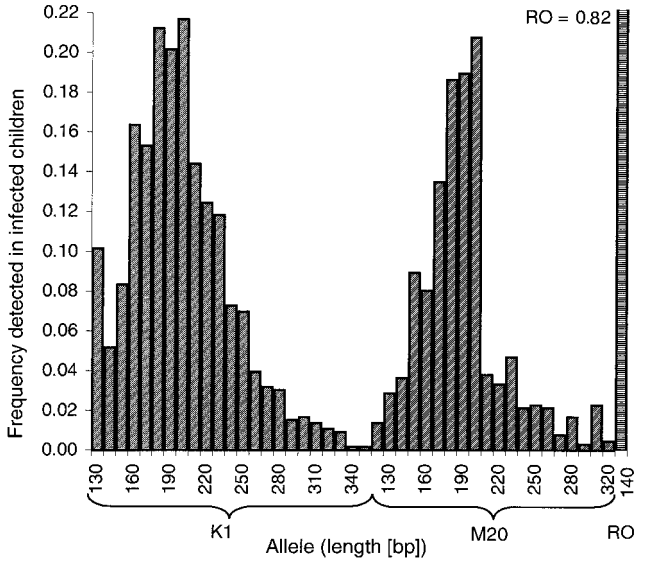

FIG. 1. Frequency of detecting MSP-1 Block2 alleles in 60 children's parasitemias from birth to 3 years of life. Using MSP-1 K1-, Mad20-, and RO-specific primers, 668 parasitemias were genotyped. There were 23 K1, 20 Mad20, and 1 RO. The frequency of detection was the number of samples (parasitemic) with the allele divided by the total samples tested. Parasitemias following a month of aparasitemia or an antimalaria treatment (isolated parasitemias) gave similar distributions (data not shown).

had the RO allele present. Sixty-five percent of infections contained all three main allelic families. The K1 main allele family was more diverse than Mad20. There were 51, 222, 225, 119, 49, and 17 samples with 0 to 5 K1 alleles, respectively (mean 1.92, SE 0.05). There were 145, 315, 154, 54, and 15 samples with 0 to 4 Mad20 alleles, respectively (mean 1.24, SE 0.04).

Contributions to COI_{KM}. Before considering the association between COI_{KM}-RO and susceptibility, we considered factors related to the COI_{KM} (Table 1). Multivariate analysis was conducted to investigate the estimated effect of malaria transmission and host immunity on the COI_{KM} in comparison to a baseline level (see above for details).

The EIR in the study area were determined (Fig. 2). Parasitemias sampled from children residing in villages with slightly higher EIR over most months had significantly higher COI_{KM} than villages with lower long-term EIR. Parasitemias in the high and medium EIR villages had an estimated COI_{KM} 0.74 and 0.59 higher than those in the low EIR villages, respectively ($P < 0.0489$). Seasonal transmission differences showed a smaller yet significant estimated effect. The COI_{KM} was estimated to be 0.28 higher in the hypertransmission season versus high-transmission season ($P = 0.0404$).

The level of host immunity could be reflected in COI_{KM}, and we investigated if the COI_{KM} was influenced by the previous month's COI_{KM}, antimalaria drug treatment, parasite density, age, sickle cell genotype, and/or sex of the child. The genotyped samples separated by <40 days were considered informative regarding level of infection and/or host immunity. This time period was characterized as parasitemic and untreated, with a certain COI_{KM}; aparasitemic with no febrile illness; or parasitemic but treated with antimalarial drugs (Fansidar). Parasitemias following untreated parasitemias with a COI_{KM} of ≥5 had an estimated COI_{KM} 0.53 greater than parasitemias following a month of aparasitemia ($P = 0.0372$). The overlapping CI of the estimated COI_{KM} when considering a previous month with a COI_{KM} of 0 to 4 showed that the only impact of a previous COI_{KM} and current COI_{KM} was when considering the most extreme case described above.

Age was significantly associated with the COI_{KM}. Children 2.00 to 3.00 and 1.49 to 1.99 years old had an estimated COI_{KM} 0.85 and 0.81 greater than children 0.00 to 0.49 respectively ($P < 0.0085$). This increased average COI_{KM} with age could not be explained by antimalaria drug treatment or EIR. However, the significant increase in COI_{KM} with age was only seen when comparing the older age groups to the children <6 months of age (a time when maternal and neonatal factors might offer some protection). There were no significant differences in COI_{KM} among the other age groups, as evidenced by the overlapping CIs.

Resistance to subsequent parasitemia. We developed an a priori definition of resistance based on all ABCP participants. The frequency of parasitemias of >500/μl decreases with age; however, the frequency of low-density parasitemias (1 to 500/μl) in children <5 years old (16.12%) is similar to that in naturally immune ABCP adults (12.3%) ($P = 0.5265$) (data not shown). This low parasitemia implies clinical immunity, since the probability of febrile illness and hemoglobin levels of <6.0 g/dl at this low parasitemia is not different from aparasitemic individuals (independent of age in this population) (Fig. 3A and B). We use the term resistant to denote resistance to parasitemia of >500/μl and any febrile parasitemia.

The time period of resistance reflected that antiparasite immunity is short-lived in children <5 years old (35–36). Eighty-two percent of the 668 infections studied had >500/μl parasitemias or a febrile parasitemia within the 2 following months. Therefore, only 18% of the children were resistant for more than 2 months following the genotyped parasitemia. After considering all the variables and potential interactions, the COI_{KM}-RO was predictive of resistance of >2 months (Table 2). Children with $COI_{KM} = 1$ were 15.2 times more likely to resist parasitemia of >500/μl and febrile illness for >2 months than when COI_{KM} was ≥5 ($P = 0.0000$). Conversely, children with the RO allele were 3.46 times more likely to resist infection for >2 months compared to children lacking the RO parasite-type ($P = 0.0012$).

Multivariate analysis was conducted to determine the variables impacting resistance to subsequent parasitemia of >500/μl and febrile parasitemia (see above for details). Of the factors reflecting host immunity, age had the greatest detected impact. The effect of age group was limited to the oldest versus the youngest age groups (Table 2). Children >2 years of age had a 15.2-fold-decreased risk of parasitemia of >500/μl or febrile parasitemia in the following 2 months than children <0.5 years old ($P = 0.0000$). The overlapping 95% CI showed that the risk of >500/μl or febrile parasitemia was similar (not associated with age, $P > 0.05$) in children 0.5 to 3 years of age.

Importantly, the association between COI and resistance was independent of age. In addition to using multivariate analysis, we found that the association was independent of age by finding a significant association between COI_{KM} and resistant for >2 months in 0.5- to 1.5- and 1.5- to 3.0-year-olds separately ($P < 0.02$ for each age group). Febrile illness, which

TABLE 1. The number of K1 and Mad20 MSP-1 Block2 alleles detected (COI_{KM}) for 683 parasitemic samples from 60 children followed for approximately 3 years, with >200/μl packed RBCs collected[a]

Variable	Variable level	Mean (SE)	Adjusted EE vs. Ref.	N Obs. (part)	95% CI of EE	*P* value vs Ref.
Village EIR	High	3.58 (0.18)	0.74	105	0.0–1.5	0.0489
	Medium	3.25 (0.08)	0.59	385	0.1–1.0	0.0090
	Low	2.79 (0.10)	0.00	193		
Seasonal EIR	Hyper	3.20 (0.09)	0.28	305	0.0–0.5	0.0404
	High	3.12 (0.08)	0.00	378		
Fever ≥37.5°C	No	3.19 (0.08)	NT			
	Yes	3.07 (0.10)				
Paras within 1 mo prior to this paras	COI_{KM} ≥5	4.40 (0.24)	0.53	50	0.1–1.1	0.0372
	COI_{KM} 4	3.54 (0.25)	0.29	48	−0.3–0.9	0.3247
	COI_{KM} 3	3.20 (0.18)	0.14	59	−0.2–0.6	0.4129
	COI_{KM} 2	2.88 (0.20)	0.09	66	−0.3–0.6	0.4799
	COI_{KM} 0–1	3.11 (0.26)	0.21	36	−0.2–0.9	0.1915
	Tx COI_{KM} 0–9	2.98 (0.23)	0.07	92	−0.3–1.5	0.1694
	Aparasitemic	2.89 (0.17)	0.00	53		
Fever within 1 mo prior	No	3.43 (0.10)	0.54	297	−0.1–1.1	0.0773
	Yes	2.88 (0.14)	0.00	121		
Age (yr)	2.00–3.00	3.67 (0.22)	0.85	95	0.2–1.5	0.0085
	1.49–1.99	3.57 (0.13)	0.81	163	0.2–1.4	0.0048
	1.00–1.49	2.83 (0.13)	0.41	143	−0.2–1.0	0.1570
	0.50–0.99	2.98 (0.10)	0.28	190	−0.2–0.7	0.2033
	0.00–0.49	2.76 (0.17)	0.00	92		
Parasite density (*n*/μl)	25,001–86,907	3.36 (0.18)	NT	72		
	10,001–25,000	3.27 (0.11)		123		
	5,001–10,000	3.43 (0.17)		105		
	2,501–5,000	3.39 (0.19)		93		
	500–2,500	3.01 (0.14)		147		
	1–500	2.76 (0.13)		130		
Sickle cell genotype	AA	3.25 (0.07)	NT	493 (43)		
	AS	3.03 (0.14)		135 (13)		
	SS	2.55 (0.18)		29 (4)		
Sex	Male	3.32 (0.09)	NT	288 (28)		
	Female	3.01 (0.09)		340 (32)		
Species detected by m.scopy	Pf and Pm	4.05 (0.28)	NT	44		
	Pf-Po-Pm	3.09 (0.58)		14		
	Pf only	2.92 (0.06)		611		

[a] The GLM controlled for repeated measurements. Variables showing no trend (NT) ($P > 0.1$) were removed. The mean, standard error (SE), and model-adjusted estimated effect (EE) are shown. The EE is relative to the reference (Ref.): set to 0. Paras = parasitemia. Tx = treated. Mo = month. Pf = *P. falciparum*. Po-Pm = *P. ovale* and/or *P. malariae*. M. scopy = microscopy. The 95% CI tests of differences ($P < 0.05$) among variable levels.

might reflect an innate (nonspecific) immune response, was associated with a threefold increase in the probability of resistance for >2 months ($P = 0.0041$), even after controlling for antimalaria drug treatment. We did not find an association between parasite density and resistance ($P > 0.5$).

We also considered if children were able to resist parasitemia of >500/μl or febrile parasitemia for 0, 1, 2, 3, or more months. The average months resistant decreased with increasing COI_{KM}, regardless of transmission or antimalarial drug treatment, while children did not resist following infections without the RO parasite (Fig. 4A to C). These correlations were significant, of a greater magnitude, and independent of the correlation between resistance and sickle cell genotype (Fig. 4D).

Although the analysis controlled for repeated measures within each individual, we wanted to determine if our results were individual specific. We found that children often alternated between the resistant >2 months and not resistant categories. Approximately 60% of the children were in the resistant >2 months category 25 to 40% of the time, suggesting that our results were not simply demonstrating contributions of host-factor-related immunity and/or genetic factors. In addition, two randomly selected sample groups, one point from each child when 0.5 years of age (near March 1993), and one point from each child when 1.5 years of age (near March 1994); each showed a significant association between COI_{KM} and resistance ($P < 0.05$).

Children were resistant following low COI_{KM}, especially

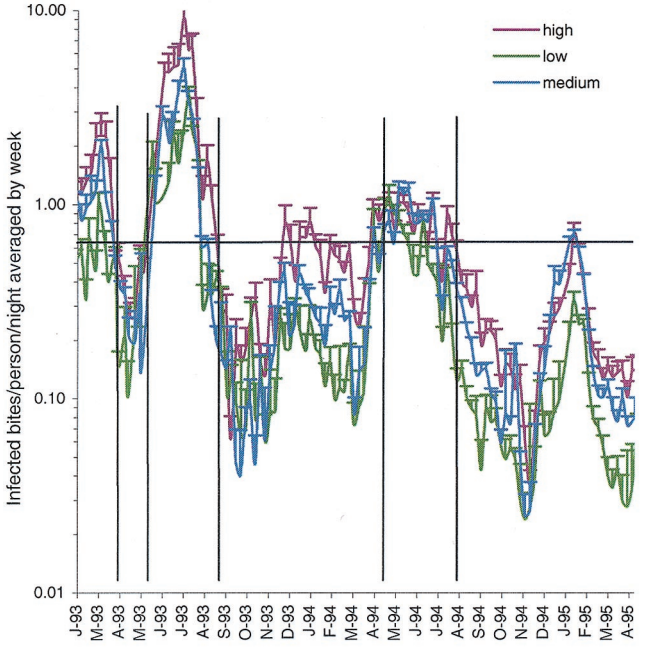

FIG. 2. Spatial and temporal EIR in Asembo Bay Cohort Project. Villages could be divided into three categories based on long-term EIR: low ($N = 4$), medium ($N = 7$), and high ($N = 4$). The means and upper SE are shown. Time periods when the average EIR were >0.5 infected bites/person/night (indicated by horizontal line) were defined as hypertransmission seasons. Season boundaries are indicated by vertical lines. J-93, January 1993, etc.

when infected with a small number of K1 alleles. However, previous genotype history or child-specific factors contributed to future parasitemia genotypes and resistance to subsequent parasitemia (Fig. 5). For instance, the child whose data are shown in Fig. 5A appeared to develop resistance to the RO parasite and, perhaps due to more effective immunity earlier in life, could remain resistant even following parasitemias lacking the RO parasite.

Considering all 60 children, the K1 alleles alternated over time even without antimalaria drug treatment (Fig. 5A to E). However, the probability of the allele's being redetected in the subsequent 2 months (approximately three blood samples) was not significantly different than expected by chance (data not shown). While, in most cases, K1 alleles were not redetected in 2 months, the same Mad20 alleles were frequently redetected.

DISCUSSION

Comprehensive, longitudinal blood sampling during the time children are highly susceptible to malaria along with detailed entomologic data enabled us to consider factors contributing to the COI, parasite populations within the child's successive infections, and the development of immunity. We estimated the overall COI by the number of K1 and Mad20 MSP-1 Block2 alleles within an infection (COI_{KM}) and the presence of the repeatless MSP-1 Block2 RO allele to test if COI_{KM} (or overall COI) was associated with either genotype-specific or genotype-transcending resistance to parasitemias of >500/μl or febrile parasitemia of any density.

The first phase of our study was to determine factors impacting the detectable COI_{KM} in this population. Our results suggest that superinfection occurring within a very short time period (<2 weeks) and/or mosquitoes inoculating more than one parasite genotype in one bite contributed to the different COI_{KM} and different genotypes detected within an individual over successive months more so than superinfection occurring over a period of 1 month. Three observations led us to this conclusion. First, the moderate village-based differences (~2-fold) in EIR had a greater impact on COI_{KM} than did vast differences in EIRs with transmission season (~10-fold). Second, parasitemias following a successful antimalaria treatment had an average COI_{KM} similar to parasitemias following untreated parasitemias of $COI_{KM} < 5$. Third, K1 "genotypes" were often not redetected in subsequent blood samples.

A child not accumulating a higher COI_{KM} over successive months and having months where few parasite genotypes were detected suggests that it is possible for children to clear parasite genotypes (to below detection level) by innate or specific immunity. In a previous Tanzanian study, the disappearance of alleles within days in asymptomatic parasitemias of <1,000/μl was attributed to sequestration (28). Sequestration and detection limits are relevant factors, especially at such low-density parasitemia. However, the large volume of blood (>200 μl of packed RBCs) used in this study, the infrequent redetection of genotypes in three successive infections, and lack of accumulating COI_{KM} over successive months suggest that children could clear parasite genotypes to below a detectable level and that the parasitemias and genotypes detected in the following

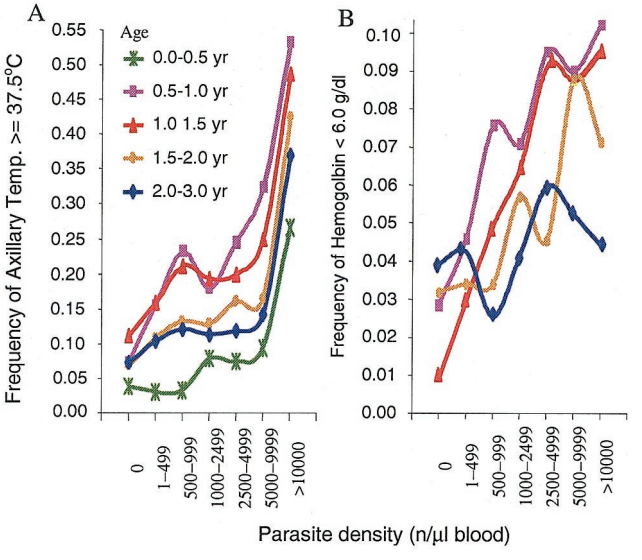

FIG. 3. Parasitemias of <500/μl were not associated with frequency of febrile illness (A) or moderate anemia (B). The routine monthly blood samples from 28, 185 ABCP children 0.0 to 0.5 (green), 0.5 to 1.0 (purple), 1.0 to 1.5 (red), 1.5 to 2.0 (orange), or 2.0 to 3.0 (blue) months old, born into the cohort, were used. Parasitemia density categories were made by binning the data. The frequencies were determined for each parasitemia group and age group. The youngest age group is only plotted in panel A due to the difficulty in assessing anemia in this age group. Parasitemias of <500/μl were not associated with increased probability of febrile illness or moderate anemia ($P > 0.05$ for each age group).

TABLE 2. Resistance to >500/μl and febrile parasitemia for >2 months following a parasitemia genotyped for MSP-1 Block2 alleles[a]

Variable	Variable level	Resist >2 mo (%)	Adjusted EE RR vs Ref.	*N* Obs. (part)	95% CI of EE	*P* vs Ref.
RO parasite	Present	19.84	3.46	535	1.6–7.3	0.0012
	Absent	13.04	1.00	119		
COI_{KM} (count)	0	50.00	26.61	16	7.1–100.0	0.0000
	1	38.78	15.21	74	5.6–41.5	0.0000
	2	33.06	8.96	155	3.3–24.1	0.0000
	3	17.73	4.63	165	1.6–13.1	0.0039
	4	10.09	2.19	122	0.7–6.9	0.1804
	≥5	3.67		121		
Village EIR	Low	22.73	NT	187		
	Medium	20.47		367		
	High	8.99		99		
Post-paras EIR exposure	High	21.44	2.88	175	1.7–3.9	0.0001
	Hyper	13.94	1.00	479		
Treatment	Yes	23.08	NT	196		
	No	17.72		458		
Fever ≥37.5°C	Yes	25.81	2.98	272	1.8–3.0	0.0079
	No	15.00	1.00	382		
Paras within 1 mo prior to this paras	COI_{KM} ≥5	12.20	NT	41		
	COI_{KM} 4	13.16		38		
	COI_{KM} 3	12.96		54		
	COI_{KM} 2	22.03		59		
	COI_{KM} 0–1	21.88		32		
	Tx COI_{KM} 0–9	20.73		81		
	Aparasitemic	17.39		46		
Fever within 1 mo prior	Yes	17.95	NT	127		
	No	16.00		288		
Age (yr)	0.00–0.49	41.77	15.24	89	4.8–48.9	0.0000
	0.50–0.99	19.65	3.74	155	1.2–11.7	0.0234
	1.00–1.49	18.32	4.93	138	1.5–15.9	0.0078
	1.50–1.99	14.69	3.82	184	1.5–9.7	0.0048
	2.00–3.00	7.32	1.00	89		
Parasite density (*n*/μl)	1–500	33.75	NT	124		
	501–2,500	16.55		141		
	2,501–5,000	18.18		88		
	5,001–10,000	10.89		101		
	10,001–25,000	19.01		121		
	25,001–86,907	23.35		71		
Sickle cell gentoype	SS	52.00	5.05	27 (4)	2.0–12.9	0.0007
	AS	23.70	1.82	141 (13)	1.0–3.2	0.0427
	AA	15.98	1.00	473 (43)		0.0000
Sex	Female	19.78	NT	325 (29)		
	Male	19.21		329 (31)		
Species detected by m.scopy	Pm and Po	15.38	NT	13		
	Pf-Po-Pm	12.50		40		
	Pf only	18.71		601		

[a] GLM controlled for repeated measurements on individuals and did not include genotyped samples within a resist >2 mo interval. Variables showing NT ($P > 0.1$) were removed. The univariate percent is shown. The EE is expressed as the adjusted relative risk (RR) relative to the Ref. (set to RR = 1). See Table 1 for abbreviations.

2 months were largely attributable to new inoculation (approximate EIR of >0.5 infected bites/person/night).

Interestingly, we found that although, as expected, children whose sickle cell hemoglobin genes were homozygous (SS) or heterozygous (AS) for the sickle cell genotype trait were more capable of resisting parasitemia of >500/μl, their mean COI_{KM} was not significantly lower than that of AA children. This observation is in agreement with Ntoumi et al., who found a higher COI in 7- to 14-year-olds with the AS genotype versus the AA genotype (32). It seems unlikely that AS or SS children

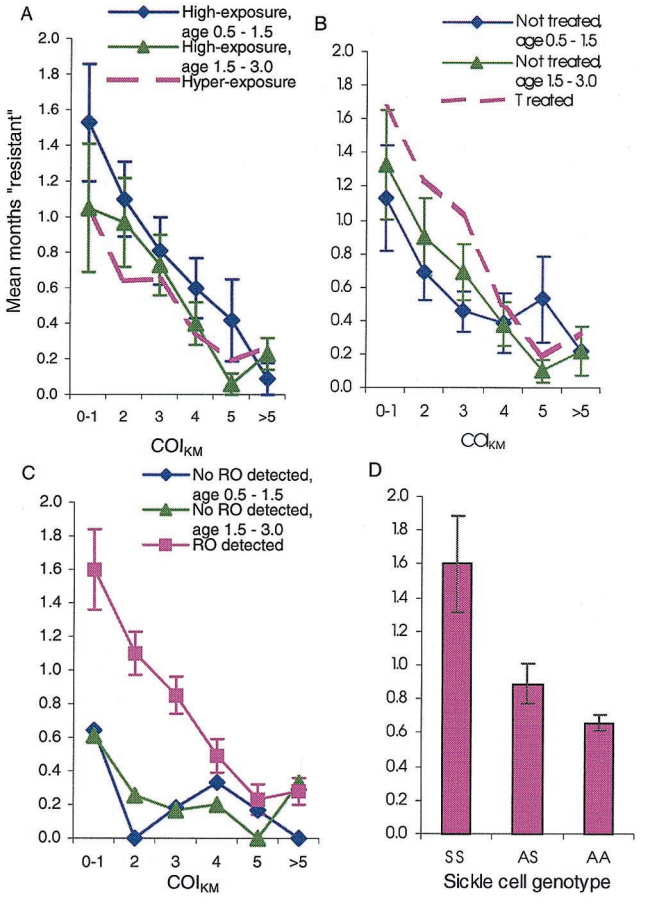

FIG. 4. COI_{KM} and RO parasitemia with the mean time of resistance to subsequent parasitemia of >500/μl and febrile parasitemia. We show the association, controlling for malaria transmission (A), administration of antimalaria drug treatment (B), absence of the RO parasite genotype (C), and sickle cell genotype (D). The results for individuals in the high-transmission and nontreated categories are stratified by age and plotted with SE bars. The parasitemias of <500/μl occurring within an interval of resistance were not genotyped and considered with regard to resistance to exclude overlap. For each stratification, $n > 15$. COI_{KIM20} and RO ($P < 0.0001$) and sickle cell ($P = 0.0031$) independently associated with months resistant.

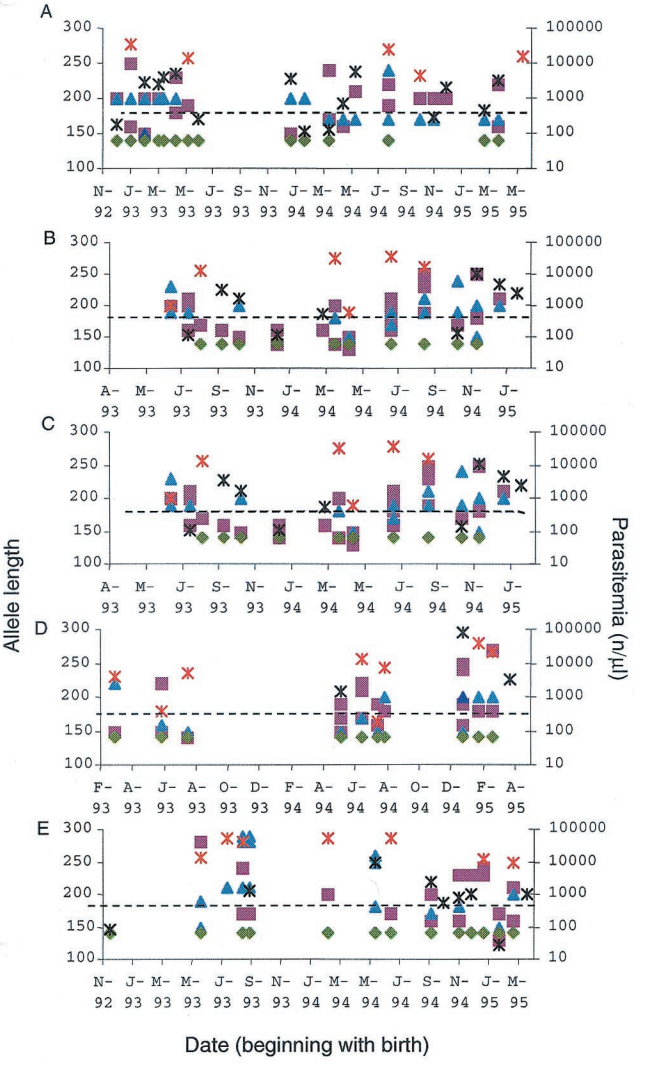

FIG. 5. Representative successive parasitemias and MSP-1 Block2 allele detection for five children (A to E). The allele lengths (K1 in red, Mad20 in blue, and RO in green) and parasite densities (black asterisk, not febrile; orange asterisk, febrile/treated) are shown. The dotted line = 500/μl. K1 alleles were often not detected in the subsequent 2 months. Children were examined in their homes every 2 weeks. Blood samples were taken at least every month. A reported or measured fever or illness resulted in an additional blood sample's being taken. Blood samples were collected during treatments (Fansidar administered at day 1, 3, and 7 postclinical malaria detection) and suggested effective parasite clearance. Periods of aparasitemia and treatment are not shown for clarity. The same K1 allele was rarely detected more than once in 2 months, but analysis showed that this was not significantly different from chance ($P > 0.05$ for any allele).

are more susceptible to infectious bites with more parasite genotypes. In comparison to AA children, AS and SS children in the ABCP appear more likely to resist parasitemia of >500/μl but not more likely to be aparasitemic (Branch et al., unpublished data). It is possible that AA children are more capable of clearing parasite genotypes or that parasites are more likely to sequester at levels below PCR detection in AA children in comparison to AS or SS children.

We found that children >2 years old had a higher COI_{KM} and a reduced ability to resist parasitemias of >500/μl for >2 months in comparison to children 0.5 to 1 year old. This contrasts with the conventional wisdom that, not considering the first 6 months, when maternal antibodies appear to provide protection, the ability of the host to control or clear malaria infections should correlate with exposure-acquired immunity (and/or age-acquired immunity, as discussed by Baird et al. [5]). In agreement with our findings, others have found a higher COI in 2- to 4-year-old children in comparison to children <2 (25). This led to the hypothesis that the fever often observed in infants could clear parasitemias, while the developing specific immune response in 2- to 4-year olds limits but does not completely clear parasite genotypes below detectable levels (25, 41, 43). The studies summarized above did not have data available on antimalaria drug treatment. Here, we find the first evidence, independent of antimalaria drug treatment, transmission, and host-related differences in susceptibility, that

febrile illness was associated with a greater likelihood of clearing and resisting subsequent parasitemia.

The facts that the COI_{KM} was not associated with parasite density and children >2 years old were less likely to resist parasitemias of >500/μl for longer than 2 months support some density-dependent regulation of parasitemias in older children (10). However, we found that, regardless of age, the previous parasitemia's COI_{KM}, transmission intensity, drug treatment, parasite density, and sickle cell genotype, >30% of the time children with a COI_{KM} of ≤3 could resist subsequent parasitemia of >500/μl for >2 months.

We considered the ability to resist subsequent parasitemia of >500/μl (or any febrile parasitemia) in two ways: the mean time of resistance and the ability to resist for ≥2 months. The former enabled us to consider the data without preset thresholds, while the latter enabled us to consider the child's having opportunity for subsequent infection in this highly endemic transmission area.

Resistance was measured as parasitemia and was not specific to any particular parasite genotype. The fact that K1 alleles often were not redetected for >2 months evokes speculation of genotype (allele)-specific immunity (22). However, our result can also be explained by the low probability of redetecting any particular genotype by chance alone. The resistance to parasitemia, regardless of genotype, suggests that genotype-transcending immunity (targeting antigenic determinants shared among most parasite genotypes) was more effective following low COI_{KM} infections with the RO parasite genotype.

Children's sickle cell trait enabled a measure of comparison of the estimated effect of COI_{KM} and RO on detected resistance in the following months. A decrease of 2 in the detected COI_{KM} was predictive of a fourfold-increased ability to resist parasitemia of >500/μl (and any febrile parasitemia) in the following 2 months. This was greater than the predicted twofold increase in resistance seen in AS children versus AA children.

The fact that MSP-1 is associated with protection, especially the highly conserved MSP-1 19-kDa fragment (19KD) (11, 15, 26, 39), is a major reason to consider MSP-1 Block2 (and loci in genetic disequilbrium with MSP-1 Block2) specifically rather than as only a marker for overall COI. There is extensive genetic disequilibrium in MSP-1, resulting in Block2 genotyping's not being indicative of the allelic types present in other regions of the gene (13). However, the exception is linkage disequilibrium between MSP-1 19KD and MSP-1 Block2 (39). The findings that the K1 allelic type was most negatively correlated with resistance and the presence of the repeatless RO parasite type was associated with greater resistance suggest that the antigenic diversity of MSP-1 Block2 (or linked antigenic diversity) impacts the development of resistance to subsequent parasitemia, but cannot rule out the impact of overall COI on the association. For instance, the K1 genotypes might be a better indication of overall antigenic diversity, since it is more polymorphic in this population, or the K1 genotypes might be more effective at antagonizing or distracting an effective immune response to Block2 or other antigenic determinants (2, 21, 29, 34, 40).

Data investigating anti-Block2's role in protection have been conflicting (7, 8, 24, 35). While some anti-Block2 antibodies are cross-reactive within main families, others are specific to differences between the main families (7, 17). Some studies suggest that high anti-Block2 antibody levels are positively associated with susceptibility to parasitemia and are not associated with protection (24, 35). In contrast, a recent study found that children with both K1 and Mad20 anti-Block2 antibodies at the end of the low-transmission season were more likely to manifest signs of clinical malaria within the following 6 months than individuals without anti-Block2 antibodies at the end of the transmission season (14). It is possible, however, that anti-MSP-1 Block2 antibodies are only a marker of recent parasitemia and, therefore, a marker of short-lived antibodies to other malaria antigens (especially MSP-1 19KD) (7, 11, 17). This would result in an association with Block2 antibodies regardless of their specific role in protection.

If anti-Block2 antibodies can impart significant protection, our results suggest that an effective response was impaired in high COI_{KM}, as predicted by the smoke screen hypothesis (2), possibly facilitated by immunologic antagonism (34). However, evoking this form of immune evasion as an explanation for susceptibility to parasitemia of >500/μl requires these postulated immune evasion mechanisms to be generalized and not specific to a particular genotype.

The association between the RO parasite and subsequent resistance, in agreement with earlier reports (1, 31), is interesting. The resistance seen following an infection with RO would be expected if RO was linked to a particular 19KD allele or an allele that did not block effective anti-19KD antibodies (42). In addition, linkage disequilibrium with other genes might result in RO parasites having different growth rates and/or erythrocyte specificity (39). Finally, and most speculatively, the lack of repeats in the RO Block2 region (and extremely limited linkage disequlibrium in *P. falciparum*) might induce a more effective immune response.

Speculation abounds regarding the role of repetitive antigens in immune evasion. It can be postulated that T-independent antibody responses to the repetitive Block2 antigens (7, 24) could result in a cytokine environment nonconducive to T-dependent antibody responses to adjacent determinants. This was first suggested and studied regarding the circumsporozoite protein repeats (40), but it was thought that the smaller Block2 would not have great potential for altering the cytokine environment. However, one must consider the natural infections where the host's immune response is exposed to many different parasite genotypes at one time (mixed infections) (33). The within-host diversity would amplify the number of B-cell clones responding within a given infection. It is important to note that homeostasis in the immune response and diverse immunogenic antigens might distract or exceed a regulation threshold (21, 29) and/or soluble Block2 might impede germinal center formation (36). Our results suggest that infections lacking the repeatless RO parasite genotype are more vulnerable to such evasion mechanisms.

Either impediment of a protective immune response to Block2 or indirect immune evasion mechanisms as described above might explain the apparent diversifying selection on MSP1 Block2 (45) and the prevalence of mixed infections (33). Some of this speculation can be directed to consider why genotype-transcending immunity, targeting conserved antigenic determinants, is delayed during natural malaria infections.

In summary, this study has provided information on dynam-

ics of parasitemia in children's first few years of life. We have found that independent of febrile illness, age, transmission, and previous months' parasitemia history, the COI_{KM} is negatively correlated with resistance to parasitemia of >500/μl in the following months. This correlation was detectable within an individual child's history of successive infections. From a public health perspective, this observation suggests that intervention control and prevention strategies that reduce the complexity of infection may lead to faster development of natural immunity that protects against high-density parasitemia and clinical manifestations of illness.

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