Use of Mosquito Preventive Measures Is Associated with Increased RBC CR1 Levels in a Malaria Holoendemic Area of Western Kenya

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Abstract. Malaria is responsible for close to 1 million deaths each year, mostly among African children. Red blood cells (RBCs) of children with severe malarial anemia show loss of complement regulatory proteins such as complement receptor 1 (CR1). We carried out this study to identify socio-economic, environmental, and biological factors associated with the loss of RBC CR1. A cross-sectional study was conducted in a malaria holoendemic area of western Kenya. Twelve socioeconomic, environmental, and biological factors were examined for a relationship with RBC CR1 level using bivariate linear regression followed by creation of a multivariate linear regression model. A significant positive relationship between RBC CR1 level and use of mosquito countermeasures was found. However, there was no evidence of a significant relationship between RBC CR1 level and malaria infection or parasitemia level. Reducing mosquito exposure may aid in the prevention of severe malarial anemia by reducing the number of infections and thus preserving RBC CR1.

INTRODUCTION

Plasmodium falciparum, a protozoan parasite transmitted by the *Anopheles* mosquito, causes an estimated 225 million cases of malaria worldwide annually.¹ Complications such as severe malarial anemia (SMA) and cerebral malaria result in close to 1 million deaths per year, mostly among African children.¹ In Africa 15% of post-neonatal mortality was attributable to *P. falciparum*.²

Our previous studies have documented that red blood cells (RBCs) of patients with SMA have acquired deficiencies in complement regulatory proteins such as the complement receptor 1 (CR1) and decay accelerating factor (CD55).^{3,4} These findings have been confirmed by others.^{5,6} Complement regulatory proteins serve to protect host cells from autologous complement activation.⁷ Accordingly, a recent cross-sectional study showed that low RBC complement regulatory protein expression was associated with complement deposition on these cells.⁸ Thus, reduced complement regulatory protein expression on RBCs could increase their susceptibility to complement-mediated lysis and phagocytosis during malaria infections. The mechanisms underlying this reduction are unknown. However, they are presumed to be related to the ability of CR1 to bind immune complexes (ICs) and carry them to the liver and spleen for removal by macrophages after repeated malaria infections.⁹ To gain greater insight into the factors that lead to RBC CR1 reductions we carried out an analysis of social, environmental, and biological factors and their relationship to RBC CR1 level in a malaria-endemic area.

MATERIALS AND METHODS

Study design. The data used for this analysis were from a cross-sectional study carried out in 2004 in the Kombewa Division, Kisumu District, western Kenya, a high intensity transmission area in the Lake Victoria basin. The study was approved by the Walter Reed Army Institute of Research Human Use Research Committee and by the National Ethical

Review Committee, Nairobi, Kenya. Informed consent was obtained from all human adult participants and from the parents or legal guardians of minors. The methodology and characteristics of the population were previously reported.⁸ Subjects were stable residents of the study area, ranging in age from 11 days to 45 years of age. Survey participants were screened for malaria infection by blood smear. Blood samples for CR1 quantitation from *P. falciparum* positive individuals were taken after successful anti-malarial treatment. The CR1 was quantitated by flow cytometry. The CR1 level was natural log-transformed to achieve a normal distribution.

Statistical analysis. The three dichotomous house construction variables were combined into a single housing factor variable. The categorical variable for type of mosquito countermeasures used was condensed into a binary variable indicating whether mosquito countermeasures were used in each subject's home. For children < 5 years of age, height and weight, as recorded through the study's clinical examination, were used to calculate a nutrition z-score based on World Health Organization (WHO) tables.¹⁰

Mapping tools available for open-source use through the internet were used to conduct geospatial analysis of exposure to *Anopheles* mosquito habitats. For each participant, distance from the nearest school to wetlands and distance from the nearest school to Lake Victoria were measured using the Quantum Geospatial Information System (Q-GIS) measurement tool as a surrogate measure of malaria exposure based on the need of *Anopheles* mosquitoes to lay their eggs in an aquatic environment.¹¹ Lake Victoria and its neighboring wetlands, man-made habitats, and temporary rain pools are common *Anopheles* breeding grounds throughout the region.¹¹ There was no data on the location of man-made and temporary pools; however, the availability of geospatial data regarding Lake Victoria and its neighboring wetlands allowed for exploratory analysis of exposure to relatively permanent natural water sources as a factor affecting CR1 level. Home location was assessed using Q-GIS software. Vector map layers representing the land area of Kenya, bodies of water, and wetlands were downloaded from the public data files of the World Resources Institute (WRI), an environmental issue think tank.^{11–15} A raster image of the study area created by the U.S. Army Medical Research Unit-Kenya was geo-referenced to the WRI maps. The coordinates

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FIGURE 1. Location of schools in relation to bodies of water.

of schools on the study area map were collected to create a delineated text point location layer. Annual rainfall totals did not vary throughout the study area, and therefore, were not used for analysis.¹⁴ Figure 1 shows the nearest school locations of the study population.

Descriptive statistics were generated for $\ln$ [RBC CR1 level] according to age group. Bivariate linear regression was performed for $\ln$ [RBC CR1 levels] on each predictor variable. Because the study population ≥ 5 years of age are at low risk for severe malaria in this region, two separate multilinear regression models were created: one for children < 5 years of age and the other for those ≥ 5 years of age. Each predictor variable was entered into a multilinear regression model, with variables removed using stepwise backward selection until only variables significant at the 0.05 level remained in the model. The under-5 regression model contained the variables for breastfeeding status and nutrition score, whereas the 5 years and older model did not. Tests of association were conducted between predictor variables to determine if there was confounding. Analysis was conducted using SAS v9.2 statistical software (SAS Institute, Inc., Cary, NC).

RESULTS

Within the data set, 12 socio-economic, environmental, and biological variables that were not previously analyzed were identified for further study. These variables are presented in Table 1. The survey sample comprised 343 participants, 211 of whom were < 5 years of age, and 132 of whom were age 5 or over. The participants were evenly distributed by sex, with 50.5% being male. Farming was the occupation for 48% of the participants or their parents, with 14% of the remaining participants identified as housewives. Regarding the participants' homes, 84% were constructed of mud as opposed to bricks, 38% of the roofs were grass-thatched as opposed to iron sheets, and 97% had unscreened or no windows.

All of the study participants lived in close proximity to permanent mosquito breeding grounds, with distance to the nearest wetland ranging from 0 to 6 kilometers and distance to Lake Victoria ranging from 1 to 11 kilometers. Sixty-six

TABLE 1
Predictor variables explored in the analysis

Variable	Definition
Socioeconomic status	
Occupation	Participant's occupation, or parental occupation for children compressed to farmer versus non-farmer
House construction material	Mud vs. brick
House roof material	Grass thatched versus corrugated iron
House window type	Screened versus unscreened or no windows
Environmental factors	
Mosquito Countermeasures	Repellent coils or netting versus no countermeasures
Home location	Approximate location indicated by name of nearest school
Biological factors	
Age	Measured in days
Sex	Male or female
Breastfeeding status	For children < 5 years of age
Height	Measured in centimeters, for children < 5 years of age
Weight	Measured in kilograms, for children < 5 years of age
Malaria status	<i>Plasmodium</i> detected by blood smear
Parasitemia level	Number of parasites per 200 white blood cells enumerated by blood smear

percent (66%) of the participants used some type of mosquito countermeasure, 48% used mosquito nets, 17% used coil repellants and/or sprays, 1% used traditional plants, and 33% used no mosquito countermeasures. The malaria prevalence among the study participants was 44%, and among the malaria positive participants, the range of parasites per 200 white blood cells was 1 to 3,515.

Among children < 5 years of age, 35% were breastfed. The mean WHO nutrition score of the participating children was 0.12 and ranged from -3.5 to 4.0 , whereas normal range is -2.0 to 2.0 , with higher scores indicating better nutrition.¹⁰

The mean CR1 level was 447 molecules per RBC (95% confidence interval [CI]: 431 to 464). Participants 18 years and older had the highest CR1 counts of 560 molecules per RBC (95% CI: 516 to 606), although the 12 to 24 months age group had the lowest at 374 molecules per RBC (95% CI: 353 to 397). Consistent with previous findings,^{4,8} analysis of variance revealed significant differences in CR1 levels based on age group ($P < 0.0001$). Figure 2 shows the pattern of $\ln$ -transformed CR1 levels by age-group.

The mean $\ln$ -transformed RBC CR1 level for the study group was 6.05 with a standard deviation of 0.32. The mean $\ln$ [RBC CR1 level] by dichotomous factor are presented in Table 2. The mean $\ln$ [RBC CR1 level] for farmers was 6.04, which was slightly lower than that for non-farmers. Participants who used mosquito countermeasures in their homes had higher CR1 levels than those who did not. The mean $\ln$ [RBC CR1 level] for females was 6.07, whereas that of males was 6.03. At 5.95, breastfeeding children had a lower mean $\ln$ [RBC CR1 level] than children who were not breastfed and whose mean $\ln$ [RBC CR1 level] was 6.03 (Table 2).

Simple linear regression of $\ln$ -transformed RBC CR1 level on each predictor variable identified two significant associations (Table 3). Use of mosquito countermeasures

FIGURE 2. Ln-transformed red blood cell (RBC) complement receptor 1 (CR1) level by age group. Data are presented as box plots where the box shows the limits of the first and third quartile, the whiskers represent the first and fourth quartile distributions, and the circles represent outliers.

was positively associated with log CR1 level ($P = 0.0004$) with an effect magnitude of 0.12, which approximately corresponds to an additional 62 molecules of CR1 per RBC. Among children < 5 years of age, breastfeeding was found to have a negative association with ln [RBC CR1 level] ($P = 0.05$). However, in light of the association between breastfeeding and a child's age, when the regression of ln-transformed RBC CR1 level on breastfeeding status was stratified by age group, no significant difference was detected.

To create a final model, the data were analyzed using the two major age groups. The under-5 years old model had an overall significance of 0.002, with female sex and use of mosquito countermeasures found to have a significant positive relationship with ln [RBC CR1 level]. The effect of female sex was 0.08, and the effect of mosquito countermeasures was 0.12, which equates to ~ 17 and 62 molecules of CR1 per RBC, respectively. Only the variable for mosquito countermeasure use remained in the ≥ 5 years old group model with an effect of 0.19 ($P = 0.0014$), which equates to ~ 100 molecules of CR1 per RBC. Table 4 provides the linear regression equations for < 5 years old model and

TABLE 2
Mean ln [RBC CR1 level] by factor*

Factor	Mean ln [RBC CR1 level]	95% Confidence interval
Occupation		
Farmer	6.04	5.99 to 6.09
Non-farmer	6.06	6.01 to 6.11
Mosquito countermeasure use		
Yes	6.09	6.05 to 6.14
No	5.97	5.91 to 6.02
Gender		
Male	6.03	5.98 to 6.08
Female	6.07	6.03 to 6.12
Breastfeeding status		
Yes	5.95	5.90 to 6.00
No	6.03	5.97 to 6.08
Malaria status		
Positive	6.02	5.98 to 6.07
Negative	6.07	6.02 to 6.12

*RBC = red blood cell; CR1 = complement receptor 1.

TABLE 3

Results of bivariate linear regression of ln [RBC CR1 level] on predictor variables*

Variable	Number observed	Parameter estimate	P value
Occupation as farmer	342	0.01	0.62
Housing factor	342	0.005	0.78
Mosquito countermeasures	343	0.12	0.0004
Lake distance (per 1 km)	331	-0.01	0.30
Wetland distance (per 1 km)	331	-0.01	0.34
Age (per 365 days)	342	0.007	< 0.0001
Sex (female)	343	0.05	0.14
Breastfeeding status	252	-0.07	0.05
Nutrition score	211	0.01	0.46
Malaria diagnosis	343	-0.04	0.17
Parasitemia level	337	-0.00005	0.32

*RBC = red blood cell; CR1 = complement receptor 1.

the ≥ 5 years old model. Further analysis revealed no significant relationship between use of mosquito countermeasures and hemoglobin levels. Additionally, mosquito countermeasures were not found to have an effect on the positive relationship between CR1 expression and hemoglobin identified in previous analyses.⁸

DISCUSSION

Protecting healthy RBCs from the immune system response is an important function of CR1. During SMA, preventing phagocytosis of healthy RBCs is a particularly critical concern.³ We hypothesized that in SMA, there is a deficiency in the complement regulatory system caused by loss of CR1 through transport and phagocytosis of ICs.³ When a sufficient amount of CR1 is stripped from a healthy RBC through transport of ICs, the RBCs may become susceptible to opsonization.³ Consistent with this view, the original analysis and publication of this data set showed that age and RBC decay accelerating factor and CR1 levels were significantly associated with C3b deposition on uninfected RBCs.⁸ Here, we aimed to expand that original work to determine if there are environmental factors associated with RBC CR1 levels. One important limitation of our study is that because the study was not originally designed to address the relationships between RBC CR1 level and environmental factors, it is possible that our analysis overlooks an unsuspected bias.

In this study, three statistically significant CR1 associations were identified. Although not a strict linear relationship, CR1 levels differed based on age group, with the lowest levels observed in children 12 to 24 months of age. In the linear regression model for participants ≥ 5 years of age, use of mosquito countermeasures was associated with an advantage of ~ 100 CR1 molecules per RBC. In the linear regression model for children < 5 years of age, the group using mosquito countermeasures had mean CR1 level 62 points higher than the non-countermeasure group, and the female group mean was ~ 17 points higher than that of males.

With normal RBC CR1 levels ranging from the 200 to 1200 molecules per RBC, it appears that the RBC population in otherwise healthy people can tolerate a substantial loss of CR1 by IC clearance before large-scale destruction of healthy cells begins; however, the question arises as to what constitutes a deficiency in CR1 level, or what constitutes a clinically significant loss of CR1. An analysis of individuals with disorders associated with loss of CR1, such as

TABLE 4
Linear regression equations for ln [RBC CR1 level]

Variable	Label	DF	Parameter estimate	Standard error	t Value	Pr > t
Intercept	Intercept	1	5.84337	0.03988	146.51	< 0.0001
Mosquito countermeasures	Not used = 0 Used = 1	1	0.12384	0.04179	2.96	0.0034
Gender	Male = 0 Female = 1	1	0.08092	0.03862	2.1	0.0373

< 5-year-old model.
Ln [RBC CR1 level] = 5.84 + 0.12 × use of mosquito countermeasures + 0.08 × female sex + e.
Model P = 0.0019.

Variable	Label	DF	Parameter estimate	Standard error	t Value	Pr > t
Intercept	Intercept	1	6.07902	0.04481	135.66	< 0.0001
Mosquito countermeasures	Not used = 0 Used = 1	1	0.18594	0.05695	3.26	0.0014

≥ 5-year-old model.
Ln [RBC CR1 level] = 6.08 + 0.19 × use of mosquito countermeasures + e.
Model P = 0.0014.

human immunodeficiency virus (HIV) and systemic lupus erythematosus, suggests that impairment begins at levels below 150 molecules per RBC.^{16,17} Although a protective genetic component to CR1 levels has been established¹⁸, accelerated loss of CR1 molecules in HIV and St. Louis encephalitis (SLE)¹⁷ allows for the possibility that environmental or other factors play a role in this process.

Surprisingly, our study showed that use of mosquito countermeasures protects against CR1 loss. Although the limitations of a cross-sectional design preclude us from reaching a definitive conclusion, there are several potential explanations for this finding. First, it is possible that mosquito countermeasures have some direct biological effect on the body. Given the varied assortment of countermeasures used, this seems unlikely. Second, it is possible that individuals who do not take precautions to limit mosquito exposure have higher antibodies against mosquito salivary gland antigens^{19,20} leading to formation of ICs. These ICs could then be processed by binding to CR1, which is removed during the transfer to macrophages.⁹ We cannot exclude this possibility. Third, and the most likely explanation in our view, is the possibility that mosquito exposure is a surrogate marker for transmission of an infectious agent. Because *P. falciparum* is by far the most common pathogen transmitted by mosquitoes in this area, this seems to be the most probable pathogen. People who are more frequently bitten by mosquitoes may lose CR1 molecules while combating subclinical infections of malaria. However, we cannot exclude the possibility that other pathogens, especially viruses such as dengue and West Nile, could be contributing to this finding. The significant positive association between mosquito countermeasures and RBC CR1 levels identified in this analysis may indicate that preservation of RBC CR1 may be an important mechanism by which limiting exposure to *Anopheles* protects children from SMA.²¹ The lack of evidence for a link between CR1 level and hemoglobin level in this study may speak against this. However, this study was not designed to detect an effect on SMA.

Also surprising was our finding that there was no relationship between malaria status or level of parasitemia and the RBC CR1 level. Together with the finding that mosquito

countermeasures are protective for loss of RBC CR1, these data suggest that acute infections are not as important as the cumulative effect of repeated infections over time. Thus, we and others have found that individuals in malaria-endemic areas have lower RBC CR1 levels than individuals in non-endemic areas.^{4,22}

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