Reduced Risk of Uncomplicated Malaria Episodes in Children with Alpha*-Thalassemia in Northeastern Tanzania

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Abstract. The prevalence of human red blood cell (RBC) polymorphisms is high in areas of intense Plasmodium falciparum transmission, and individuals carrying these genetic traits are believed to be partially protected against severe malaria. However, it remains uncertain how RBC polymorphisms affect the susceptibility to uncomplicated malaria. We compared the risk of suffering from febrile, uncomplicated malaria between individuals carrying three common RBC polymorphisms (sickle cell trait, alpha*-thalassemia, and glucose-6-phosphate-dehydrogenase deficiency) and controls. The study was performed in an area of intense malaria transmission where 202 individuals 0–19 years of age were monitored clinically for a period of 6 months. RBC polymorphisms were assessed with molecular methods, and plasma antibodies to P. falciparum variant surface antigens (anti-VSA IgG) and glutamate-rich protein (anti-GLURP IgG) were measured with flow cytometry and ELISA assays, respectively. Regression analyses showed that alpha*-thalassemia was associated with a reduced risk of uncomplicated malaria episodes and that this advantageous effect seemed to be more predominant in children older than 5 years of age, but was independent of levels of antibodies to VSA and GLURP.

INTRODUCTION

Individuals living in malaria-endemic areas, repeatedly exposed to Plasmodium falciparum infections, are partially protected from clinical malaria episodes by various mechanisms of acquired immunity.1 In addition, a variety of inherited genetic traits, conferring a level of innate immunity, has been selected in populations under high malaria pressure.2–4 The most studied traits are the human red blood cell (RBC) polymorphisms, of which structural hemoglobin variants (HbS, HbC, and HbE), thalassemias (α and β), and glucose-6-phosphate-dehydrogenase (G6PD) deficiency are widespread in present or former malaria-endemic areas.5,6 These traits have all been found to be associated with reduced susceptibility to severe malaria.4,7–9 Individuals with sickle cell trait (HbAS) also seem to be protected against uncomplicated malaria and parasitemia.10–12 but data for the relationship between uncomplicated malaria and alpha*-thalassemia or G6PD deficiency are conflicting. Some studies have reported that the incidence of uncomplicated malaria is unaltered or slightly reduced,4,13,14 whereas other studies have indicated that the incidence is increased.15,16

It remains largely unknown how the different RBC polymorphisms affect malaria susceptibility. Among suggested effector mechanisms are impairment of parasite invasion and growth of RBCs,17,18 enhanced phagocytosis and premature removal of infected RBCs,19,20 and acquisition of effective immune responses.21,22 It has been suggested that cellular immune mechanisms could protect individuals with thalassemia and sickle cell trait23,24 and that alterations of the RBC membrane lead to antibody and immune opsonisation reactions.21,25 Increased levels of immunoglobin G (IgG) with specificity for variant surface antigens (anti-VSA IgG) antibodies have been observed in HbS and HbC individuals,22,26,27 whereas other studies have failed to detect higher levels of malaria specific IgG antibodies in HbS or G6PD-deficient individuals.28–33

The aim of our study was to examine whether individuals carrying sickle cell trait, alpha*-thalassemia, or G6PD deficiency have a reduced risk of suffering from uncomplicated malaria and whether such protection could be explained by increased levels of anti-malarial IgG. In a 6-month longitudinal study, we collected morbidity data in a cohort of 0.5- to 19-year-old individuals living in a high-transmission area in northeastern Tanzania and measured the plasma levels of anti-malaria IgG against P. falciparum variant surface antigens (VSAs) and conserved glutamate-rich protein (GLURP) at the beginning and end of the study.

MATERIALS AND METHODS

Study site and populations. As part of an immunoepidemiologic survey of malaria, a 6-month longitudinal study was conducted between April and September 2001 in Mgome village in Tanga region, northeastern Tanzania. Malaria morbidity was assessed by active and passive case detection as described in detail elsewhere.34 The Mgome village is situated in an area with year-round intense transmission of P. falciparum. For this study, paired samples of plasma and filter paper blot spots were collected in April and September (before and after the peak malaria transmission season, respectively) from 202 children between 0.5 and 19 years of age. The point prevalence of P. falciparum parasitemia at the beginning of the study in April 2001 was 81% (N = 250) and varied only slightly during the study period. Parasite densities and levels of hemoglobin were as previously described.34 An episode of uncomplicated malaria was defined as a patient presenting with an axillary temperature ≥ 37.5°C and/or a history of fever within 48 hours and a positive blood slide with asexual parasites > 5,000/μL. Children were grouped as "no cases" if no malaria was detected and grouped as "cases" if they presented with one or more malaria episodes during the 6-month follow-up period. After an episode, the study participant was censored the following 4 weeks to ensure that the infection caused by this malaria episode was only recorded.
once. Written informed consent was obtained from all study participants or from his/her parent or guardian. Ethical approval was granted by the Medical Research Coordinating Committee, Dar Es Salaam, Tanzania.

**DNA extraction and genotype screening.** DNA was extracted from filter papers in a 96-well plate format using Chelex-100 as described previously. Sickle cell trait and G6PD deficiency were determined by screening DNA for single nucleotide polymorphisms (SNPs) in the β-hemoglobin (A18T) and G6PD (G202A) genes by a simple high-throughput method using polymerase chain reaction (PCR), sequence-specific oligonucleotide probes (SSOPs), and ELISA-based technology. Individuals with no β-hemoglobin A18T mutation were HbAA, those heterozygous for the mutation were HbAS (sickle cell trait), and those homozygous for the mutation were classified HbSS (sickle cell disease). Individuals with no G202A mutation were classified G6PD B, those heterozygous for the G202A mutation (G6PD A/A− or B/A−) were classified G6PD A, and those homozygous or hemizygous (males) for the G202A mutation were classified G6PD A−, the G6PD deficiency phenotype. The prevalence of alpha+-thalassemia was determined by detection of the African α-globin deletion, α3.7, by PCR as described previously.

**Antibody assays.** The plasma IgG antibody level to GLURP (purified his-tag recombinant R0-GLURP) was measured by ELISA as reported in detail previously. The plasma level to VSAs expressed on erythrocytes of six in vitro–cultured parasite isolate was measured by flow cytometry as previously described. The parasites were obtained from randomly selected children living in Mgome village who carried an asymptomatic infection during the cross-sectional survey in April 2001. Each isolate had a unique genotype as determined by mspl and mspl2 PCR analysis. The geometric mean fluorescence index (MFI) was recorded as a measure of anti-GLURP IgG reactivity with specificity for each particular parasite isolate. Non-specific labeling was evaluated by analysis of ethidium bromide–negative RBCs.

The cut-off for a positive anti-GLURP and anti-GLURP IgG response, respectively, was defined as an antibody level above the mean level plus 2 SD of a group of negative control plasma samples from Danish donors never exposed to malaria. Because all children had anti-GLURP antibodies against the tested isolates, the median of VSA IgG antibody levels were used to group children into “low” and “high: responders, respectively.

**Statistical analysis.** All data were double-entered and analyzed statistically using Stata/SE version 8 and SigmaStat version 3.0. Differences in proportions were analyzed using the χ² test or Fisher exact test. Associations between independent variables (RBC polymorphisms as binary variables) and dependent variables (presence of febrile episodes and means of hemoglobin levels, parasite densities, and antibody levels) were determined by logistic regression models, adjusting for the confounding effect of age and sex. Levels of parasite densities and antibodies were normalized by log transformation. Comparison of linear or log-transformed mean values between two or more groups were made with the Student unpaired t test and ANOVA test, respectively. The non-parametric Mann-Whitney rank sum test was applied when data were not normally distributed. P < 0.05 was considered statistically significant.

**RESULTS**

General characteristics of the children in April 2001 are summarized in Table 1. The data from the survey in September 2001 did not differ significantly with respect to variables studied or differences reported for other than the variables mentioned below.

**Frequency of RBC polymorphisms.** Sickle cell trait (HbAS) was present in 14.9% of the children. No child was homozygous HbSS. The frequency of alpha+-thalassemia was 44.6% (heterozygous) and 9.7% (homozygous), resulting in a carriage rate (heterozygous + homozygous) of 54.3%. The frequency of G6PD deficiency was 10.7% (heterozygous) and 5.1% (homozygous), resulting in a carriage rate (heterozygous + homozygous) of 15.8%. All genotype polymorphisms were equally distributed among different ages and between males and females, with the exception of heterozygous G6PD (X-linked gene), which was not present in males (data not shown). Seven samples could not be amplified for assessment of alpha+-thalassemia, and six samples could not be amplified for assessment of the G6PD genotype.

**Associations between RBC polymorphisms and parasitemia and anemia.** Regardless of age, the proportion of children with parasite infection in April 2001 was lower in children with sickle cell trait than in children without (χ² = 5.32, P = 0.02), but this was not the case in September 2001 (χ² = 0.83, P = 0.36). No differences in parasite prevalence rate were found with respect to alpha+-thalassemia or G6PD deficiency. Similarly, geometric mean parasite densities tended to be lower in children with HbAS compared with children with HbAA (P = 0.07), whereas associations between parasite densities and alpha+-thalassemia or G6PD deficiency were not found in April (Table 1) or September (data not shown).

Children homozygous for alpha+-thalassemia were at a higher risk of being mildly anemic (hemoglobin levels < 11 g/dL) in both April (OR = 0.27; 95% CI = 0.08–0.96; P = 0.04) and September (OR = 0.25; 95% CI = 0.08–0.84; P = 0.03, both adjusted for the confounding effect of age). This was not the case in children with sickle cell trait or G6PD deficiency.

**Associations between RBC polymorphisms and febrile episodes.** During the 6-month study period, 176 of 202 children adequately completed the clinical follow-up. Among these, 56 (32%) had one or more febrile malaria episodes confirmed by malaria microscopy (parasites > 5,000/μL). The risk of developing a febrile malaria episode decreased with age (OR = 0.67; 95% CI = 0.58–0.79; P < 0.001). Logistic regression models adjusting for the confounding effect of age showed a reduced risk of febrile malaria episodes in children with alpha+-thalassemia. The decreased susceptibility in children with alpha+-thalassemia was significant both for heterozygous (OR = 0.30; 95% CI = 0.10–0.85; P = 0.02), homozygous (OR = 0.12; 95% CI = 0.02–0.43; P = 0.03), and homozygous and heterozygous combined (OR = 0.26; 95% CI = 0.09–0.71; P = 0.008; Table 2). The risk was mainly reduced in children older than 5 years of age (Table 3). No statistically significant reduction in malaria risk could be detected for any of the other RBC polymorphisms, and the conclusions did not change when the regression models also were adjusted for the presence of other genotypes (i.e., HbAS and G6PD deficiency).
**Associations between RBC polymorphisms and antibodies.** We assessed the association between RBC polymorphisms and malaria antibodies by comparing children with or without RBC polymorphisms with respect to the presence and levels of plasma IgG antibodies against recombinant GLURP-R0 and VSA expressed by six heterologous parasite isolates.

No significant differences in levels of anti-GLURP IgG or anti-VSA IgG (antibody levels against the six parasite isolates tested independently and in total) were detected for any of the RBC polymorphisms in either April (Table 1) or September. The level of anti-VSA IgG (Figure 1A) and anti-GLURP IgG (Figure 1B) in April increased by age both in children with and without alpha+-thalassemia. A similar tendency was found for antibody levels in the September survey. Furthermore, in logistic regression models adjusting for age and parasite density in the April survey, no significant differences in the likelihood of having a measurable anti-GLURP IgG response could be detected between children with or without sickle cell trait (OR = 2.65; 95% CI = 0.51–13.8; P = 0.37), alpha+-thalassemia (OR = 0.65; 95% CI = 0.26–6.4; P = 0.24), or G6PD deficiency (OR = 1.09; 95% CI = 0.87–1.36; P = 0.45). Similarly, there was no statistically significant associations between anti-VSA IgG in children with or without the sickle cell trait (OR = 0.68; 95% CI = 0.01–3.39; P = 0.26), alpha+-thalassemia (OR = 0.70; 95% CI = 0.19–2.58; P = 0.59), or G6PD deficiency (OR = 1.06; 95% CI = 0.88–1.27; P = 0.54). The levels of anti-GLURP and anti-VSA IgG were not influenced by the density of parasites. Logistic regression models with data from the September survey gave similar results as the data obtained from the April survey.

**DISCUSSION**

In this study, we observed a reduced risk of uncomplicated malaria episodes in children homozygous and heterozygous for alpha+-thalassemia, and the protective effect of alpha+-thalassemia was most pronounced in children older than 5 years of age. In line with other studies, we found that alpha+-thalassemia was associated with lower hemoglobin levels but not lower parasite densities.  

The protective effect of alpha+-thalassemia against severe forms of malaria has been well described, whereas its role against uncomplicated disease is less clear. In Papua New Guinea, mild malaria incidences were not reduced in children with alpha+-thalassemia, whereas a study from Vanuatu reported that alpha+-thalassemia was associated with even higher incidences of uncomplicated malaria (mainly *P. vivax*) and that this was more pronounced in the youngest children. In line with our results, children with alpha+-thalassemia were associated with reduced risk against also uncomplicated malaria in studies from Kenya (although not significantly) and Nepal. The reasons for the discrepancy in results between the different studies could be because of differences in transmission intensity, case definitions, or age groups included.

The observed age-specific protective effect suggests that the reduced risk is mediated through an interaction between immune processes and the innate mechanisms of the genetic trait, which allows the immune system to control malaria earlier or more effectively in children with alpha+-thalassemia as suggested for individuals with hemoglobin S and C. This
Notion is not contradicted by a recent study suggesting that the protective effect of sickle cell trait against severe anemia and all-cause mortality mainly was found in children 2–16 months of age,7 because it is in this age group that children acquire immunity to these malaria syndromes. Our results also showed that children younger than 5 years of age with alpha+-thalassemia were almost as susceptible to uncomplicated malaria as children without alpha+-thalassemia. One could speculate that alpha+-thalassemia is an advantage against severe malaria predominantly in infants (<2 years), whereas its protective effects against uncomplicated malaria would be more pronounced in older children (>5 years) on improved immunity, as shown for the sickle cell trait.43 However, based on the small number of cases and the limited sample size, any conclusions based on the age-stratified analysis should be made with caution.

To study whether this apparent partial protection might be related to acquired humoral immunity, we measured antimalarial antibodies with specificities that have previously been associated with protective immunity.44,45 We hypothesized that the protective effector mechanisms against severe and uncomplicated malaria are functionally similar, and therefore, that modifications of RBC membranes in individuals with alpha+-thalassemia could imply an increase of antibody binding and consequently improvement of immune clearance against both severe and uncomplicated malaria. A similar mechanism has been proposed in RBCs expressing hemoglobin C.35 Several studies have identified high levels of antibodies to both GLURP44,46 and VSA against homologous and heterologous parasite isolates47,48 as predictors of reduced parasite density and malaria morbidity, and these targets were therefore chosen for analysis. However, in this study, we did not find any significant associations between increased levels or prevalence of IgG antibodies to VSA or GLURP and decreased malaria susceptibility in children with alpha-thalassemia.

One explanation for this could be that antibody responses to other P. falciparum antigens than those measured are of importance, but it could also be that there are differences in the fine specificity or the functional capacity of the antibodies between the children with and without alpha+-thalassemia. Alternatively, alterations of the RBC membrane associated with RBC polymorphisms could enhance cellular immune mechanisms, and distinctive distributions of dendritic cells and monocytes associated with sickle cell trait and alpha+-thalassemia could contribute to protection.49

The prevalence of parasites were lower in children with sickle cell trait, supporting its protection against high parasitemia as shown by others.12,26 However, in contrast to previous findings,10,11,29,43 it was not associated with reduced risk of uncomplicated malaria in our study. We did not observe differences in GLURP and VSA antibody levels between children with and without HbAS. This contrasts previous observations showing that children with HbAS had higher levels of anti-VSA IgG,22,27 but is in line with other studies that also failed to show differences in malaria antibody levels between children with or without HbAS.28–32 Recently Verra and others26 showed that carriers of HbS and HbC in a low-transmission area, had higher immune responses to a variety of malaria antigens. They suggested that this could reflect a saturated immunity in individuals living under high malaria exposure, and this might also explain why we did not detect a difference in antibody prevalence in this study. The hypothesis that antibodies mediate protection in individuals with RBC polymorphisms was further challenged by Sarr and others,53 who observed lower levels of IgG antibodies in children with HbAS and G6PD deficiency to the merozoite antigens MSP2 and RESA. In our study, children with G6PD deficiency did not show a reduced risk of uncomplicated malaria or higher levels of malaria antibodies, which is in line with previous findings.4,53

There could be several reasons for not detecting differences in antibody response between the groups: 1) the limited sample size diminished the statistical power to detect an effect of the polymorphisms and their relation to significant differences in antibody responses; 2) antibodies against VSA on parasites causing severe malaria might correlate better with protection than antibodies against parasite isolates expressing
VSAs characteristic for uncomplicated malaria; 3) considering short-term fluctuations in antibody levels, measuring antibody responses only twice, irrespective of the clinical status of the child at the time of blood sampling, might not adequately reflect the actual immune status of the child.

We showed that the frequency of alpha⁺-thalassemia in this study area is higher than the frequency of HbS and that it correlates strongly with malaria endemicity. A reason for not observing an advantageous effect of HbAS against uncomplicated malaria in this population could be because of the low frequency of the HbS allele, which did not allow for such studies. This low frequency could again be explained by
the dominance of the alpha+-thalassemia polymorphism. Recently, it was predicted that, although alpha+-thalassemia and sickle cell trait independently were associated with resistance to clinical malaria, their protective effect was diminished when co-inherited.51

In conclusion, our findings suggest that alpha+-thalassemia is associated with protection against episodes of uncomplicated malaria, and this advantageous effect seems to be more predominant in children older than 5 years of age but seemed unrelated to antibody responses against VSAs and GLURPs.

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