The Role of Red Blood Cell Polymorphisms in Resistance and Susceptibility to Malaria

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In regions highly endemic for *Plasmodium falciparum* malaria, red cell polymorphisms that confer resistance to severe disease are widespread. Sickle cell trait, α-thalassemia, glucose-6-phosphate dehydrogenase deficiency, and blood groups were determined in 100 children from Gabon with severe malaria who were matched with 100 children with mild malaria and followed up for evaluation of reinfections. The sickle cell trait was significantly associated with mild malaria and blood group A with severe malaria. During follow-up, the original severe cases had significantly higher rates of reinfection than the original mild cases, with higher parasitemia and lower hematocrit values. Incidence rates did not differ in the context of erythrocyte polymorphisms, but patients with sickle cell trait presented with markedly lower levels of parasitemia than those without. Thus, the severity of malaria is partly determined by the presence of blood group A and the sickle cell trait. The different presentation of reinfections in severe versus mild cases probably reflects different susceptibility to malaria.

In regions highly endemic for *Plasmodium falciparum* infection, the majority of children with malaria present with a mild form of the disease. Only a small percentage of those infected go on to develop severe disease and subsequently die of it [1]. This is attributed in large part to host resistance factors that have developed during the several thousand years of high exposure to *P. falciparum* malaria [2]. Since children who are susceptible to severe malaria die before reproductive age, genes that confer resistance to severe disease should be widespread in the population living in sub-Saharan Africa. Genetic polymorphisms of the innate immune system and of the erythrocyte have thus been proposed as factors protecting against severe malaria [3].

The protective effect of certain red cell polymorphisms, such as hemoglobin (Hb) S (HbS), α-thalassemia, β-thalassemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency, hemoglobin E, and ovalocytosis, against severe *P. falciparum* malaria has been suggested because epidemiological evidence links the distribution of these polymorphisms to areas currently or historically highly endemic for malaria [4].

While direct evidence through case-control studies has confirmed the protective role of the sickle cell trait, the findings are equivocal in the case of other erythrocyte polymorphisms. We therefore decided to evaluate the polymorphisms most frequently encountered in our study area (α-thalassemia, G6PD deficiency, HbS, and blood groups) and their role in development of malarial anemia and hyperparasitemia. These are the most frequent reasons for hospital admission in our setting, whereas organ complications such as cerebral malaria are much rarer.

If certain children are in some way innately resistant or susceptible to malaria, they should show different patterns of infections, which would be detected only by long-term follow-up. This information would be missed in cross-sectional surveys, whereas the few longitudinal studies conducted to date have the weakness of having included too few, if any, severe cases.

We therefore decided to investigate patients with severe malarial anemia and hyperparasitemia, matched with patients without anemia and with low parasitemia. After treatment, these individuals were admitted to a prospective study, allowing us to evaluate associations between reinfection rate and the original disease severity and to compare erythrocyte polymorphisms with disease severity on admission as well as the rate and severity of reinfections.

Patients and Methods

The study took place at the Research Unit of the Albert Schweitzer Hospital, in Lambaréné, Gabon, where *P. falcipa-*

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Informed consent was obtained from all parents or guardians of the study children. The study was approved by the Ethics Committee of the International Foundation of the Albert Schweitzer Hospital. The study was conducted according to the human experimentation guidelines of the committee.

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rum malaria is hyperendemic [5]. From 1995 to 1996, all children with severe malaria living in Lambaréné or the vicinity were consecutively admitted into the study. Severe malaria was defined as hyperparasitemia (>250,000 parasites/μL, corresponding to >10% infected erythrocytes) and/or severe anemia (Hb of <50 g/L), as well as other signs of severe malaria [6]. Within the shortest time possible, a patient with severe malaria was matched with a child of the same sex and age who was living in the same area but had mild malaria. The criteria for mild malaria included the following values: parasitemia of 1,000–50,000 parasites/μL on admission; Hb, >80 g/L; platelets, >50/nL; leukocytes, <12/nL; lactate, <3 mM; glucose, >50 mg/dL; and schizontemia, zero. Patients with a history of hospital admission were not included in the mild-malaria group because of the uncertainty that they had never experienced severe malaria. Patients suffering from sickle cell anemia or chronic diseases or who had taken antimalarials within the preceding week were excluded [6].

Patients with severe malaria were hospitalized and received a 4-day course of quinine and clindamycin and supportive therapy as needed. Mild cases were treated with a single dose of sulfadoxine/pyrimethamine. After treatment the children became part of a prospective study to evaluate the role of immunologic, socioeconomic, and parasitologic factors in malarial infections. Each child was seen at least every 2 weeks, when a thick blood smear was made, the rectal temperature was recorded, and parents were asked about the health of the child. The parents were urged to visit the hospital if they noted any symptoms of disease. A reinfection was defined as parasitemia with *P. falciparum* accompanied by malaria symptoms.

All parents gave informed consent. The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital.

Hb type was determined by acetate cellulose electrophoresis. The α-thalassemia genotype was determined with a PCR amplification strategy with three primers [7], analyzing the 3.7-kb deletion, since it is the only form of α-thalassemia described in Central Africa [8] and the 4.2-kb deletion was not found in a control group of 121 patients in the study area (authors' unpublished data). G6PD alleles were analyzed by PCR amplification and subsequent hybridization. Since the G6PD A and B variants have almost the same enzyme activity, the patients were stratified into groups with heterozygous, hemizygous, and homozygous G6PD A− genotype.

Statistical analysis was performed with Wilcoxon's signed-rank test and the McNemar test for the cross-sectional part of the study. For the longitudinal survey, incidence densities were calculated by dividing the number of reinfections by the time of follow-up in years. These data were compared by Kaplan-Meier survival analysis with use of the log-rank test for comparisons. In cases of reinfection, the time point was set to zero and a new observation period was begun. This allowed an evaluation of every reinfection rather than just one reinfection per person [9]. The Mann-Whitney *U* test and Student's *t* test were used to compare parasitemia and hematocrit values, respectively.

Table 1. Red cell polymorphisms and severity of malaria on admission.

Polymorphism	No. of patients: Severe malaria (n = 100)	No. of patients: Mild malaria (n = 100)	P value	OR (95% CI)
Sickle cell trait	10	21	.04	2.3 (1.1–5.4)
Blood group A	27	11	<.01	0.3 (0.2–0.7)
Blood group O	54	64	.21	1.5 (0.9–2.7)
α-Thalassemia				
Normal	51	46	.35	1
Heterozygous	37	43	.35	1.3 (0.7–2.3)
Homozygous	10	10	.82	1.1 (0.4–2.9)
G6PD deficiency				
Normal	70	69	1.00	1
Heterozygous	19	19	.85	1.0 (0.5–2.1)
Homozygous or hemizygous	10	10	.82	1.0 (0.4–2.6)

NOTE. Three patients could not be typed for α-thalassemia or for G6PD deficiency.

Results

Cross-Sectional Survey

The two groups consisted of 61 females and 39 males. The average age was 44 months. The detailed clinical and laboratory data have been published elsewhere [6]. In brief, there were 83 cases of hyperparasitemia and 39 cases of severe anemia, and 73 patients had a hematocrit value of <25%. Nine children had cerebral malaria; all of them had either hyperparasitemia or severe anemia in addition.

There were 10 patients with sickle cell trait in the severe group, compared with 21 in the mild group (table 1). This difference was statistically significant, with an odds ratio of 2.3 (P = .04). Blood group A was significantly associated with severe malaria; it was noted in 27 patients in the severe group and 11 in the mild group (OR, 3.0; P < .01). Blood group O was seen more often in the mild group, although this difference was not statistically significant.

The gene frequency of α-thalassemia was 0.30. No association could be found between genotype and disease severity, and the distribution was very similar in both groups. The same is true for G6PD deficiency: the two groups contained almost the same number of phenotypes, and differentiating between the B, A, and A− genotypes or between males and females did not significantly change the even distribution. The gene frequencies of the B, A, and A− alleles were 0.59, 0.21, and 0.21, respectively.

There was no difference between the different polymorphisms and either the hematocrit or parasitemia value on admission.

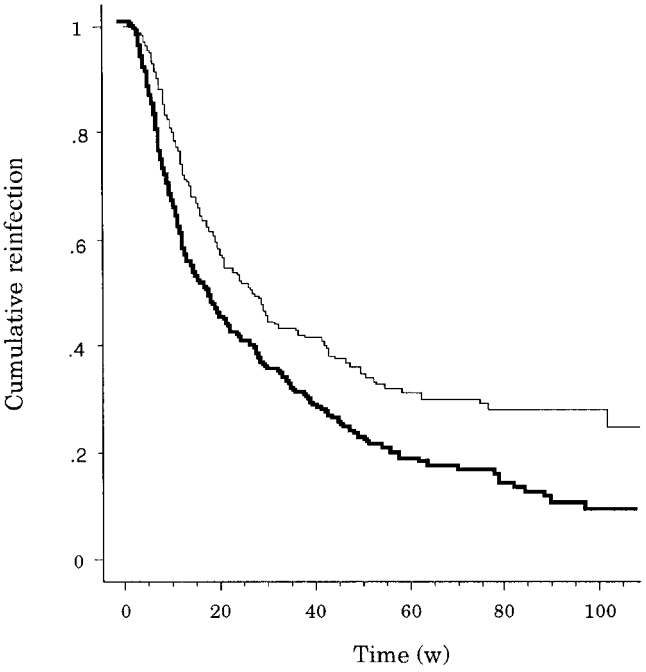

Figure 1. Analysis of not being reinfected over a period of 2 years, for patients who originally had severe (▬) or mild (—) malaria ($P < .001$).

Longitudinal Survey

The children were followed for a total of 291 person-years, and 378 reinfections were recorded up to the time of analysis, giving an overall incidence rate of 1.3 infections per child per year. However, this was subject to great variability: whereas, for example, 10 patients had eight or more reinfections each during a total follow-up of 23 years (incidence rate, 4.1), the same number of patients was not reinfected during approximately the same time period.

A striking difference was found when comparing reinfections in patients who had originally presented with severe malaria and those in patients with mild malaria (figure 1). Patients who were admitted to the study with severe malaria had reinfections at significantly higher incidence rates than did the patients who originally had mild malaria ($P < .001$), and they presented with lower hematocrit values upon reinfection ($P = .04$). Furthermore, the parasitemia values in reinfections were higher for the patients who originally had severe malaria, although this difference was not statistically significant ($P = .09$) (table 2).

The incidence of reinfections among children heterozygous for G6PD deficiency was higher than among G6PD-normal females ($P = .03$) or G6PD-normal males and females ($P = .03$). No association between carriage of the G6PD A− allele and hematocrit or parasitemia values with reinfections were found in either sex in comparison with G6PD-normal patients.

Survival analysis of not being reinfected showed no difference among patients with any other erythrocyte polymorphism, including HbS (figure 2).

However, differences were found in parasite densities and hematocrit values in the reinfections: in patients with HbAS, the median level of parasitemia was almost one-fifth that of those with HbAA ($P < .01$). Reinfections in children homozygous for α-thalassemia showed a trend toward higher levels of parasitemia ($P = .07$), and the hematocrit values associated with these reinfections were also lower than in those with normal hemoglobin ($P < .01$).

Discussion

The most surprising and interesting finding of the study was the different presentations of the reinfections in children who had had severe disease and in those who had never had severe malaria. Not only was the incidence rate of reinfections higher in severe cases, but also their reinfections were associated with lower hematocrit values and higher parasitemia levels. Different rates of transmission could in part account for the difference in incidence rates. However, the differences in hematocrit and parasitemia values, which were not as pronounced, cannot be easily explained by differences in transmission, as all reinfections were treated immediately.

In a pilot study on mosquito biting rates in a selection of these children, no differences were found (authors' unpublished observations). Other studies correlating transmission rates with clinical severity of malaria showed equivocal results [10, 11]. In addition, socioeconomic factors had an influence on neither disease severity nor time to first reinfections [12]. We feel that another but not mutually exclusive explanation can better account for the results, namely, that the different reinfection rates reflect partial innate resistance to malaria. In fact, in the same group of patients, we found an association between a prolonged time to first reinfection and a point mutation in the promoter region of the inducible nitric oxide synthase gene ($NOS2^{Lambaréné}$) [13].

Even though the mechanism remains unclear, the only red cell polymorphism associated indisputably with protection against severe malaria is the sickle cell trait, and the difference in prevalence of HbAS found in this study was therefore expected. The odds ratio of 2.3 we found is low compared with that found in similar studies in The Gambia [14] and Nigeria [15], in which odds ratios of 13.0 and 5.3, respectively, were calculated. This may be due to different clinical manifestations of severe disease, since a high percentage of patients in The Gambia and Nigeria suffered from cerebral malaria, which is rare in Gabon.

The role of sickle cell trait in mild disease is still uncertain. Studies in Nigeria [16] and The Gambia [17] have shown similar parasite prevalences but significantly lower parasite densities in subjects with HbAS than in those with HbAA, whereas studies in Uganda [18] and Liberia [19] also found

Table 2. The incidence rates and parasitemia and hematocrit values for reinfections, segregated according to clinical presentation on admission and red blood cell polymorphisms.

Feature	*n**	IR†	*P* value‡	Parasitemia§ (95% CI)	*P* value	Hematocrit# (95% CI)	*P* value
Severe malaria	244	1.39	. . .	30,000 (20,000–50,000)	. . .	31.1 (30.4–31.9)	. . .
Mild malaria	134	0.51	<.001	26,000 (11,000–40,500)	.09	32.3 (31.5–33.2)	.04
Hemoglobin AA	333	0.91	. . .	33,000 (24,000–48,500)	. . .	31.5 (30.9–32.1)	. . .
Hemoglobin AS	45	0.62	.28	7,200 (2,500–17,500)	<.01	31.9 (30.5–33.2)	.68
Blood group A	71	0.88	.85	50,000 (12,500–90,000)	.07	31.4 (30.0–32.9)	.83
Blood group 0	223	1.08	1.0	37,000 (24,000–50,000)	.14	31.5 (30.8–32.3)	.97
α-Thalassemia							
Normal	177	1.19	.63	25,000 (10,000–45,000)	.53	32.5 (31.7–33.3)	<.001
Heterozygous‖	145	0.80	.98	30,000 (15,000–42,500)	.96	30.9 (30.0–31.8)	<.01
Homozygous**	53	0.90	.17	50,000 (20,000–90,000)	.07	29.7 (28.0–31.4)	.01
G6PD deficiency							
Normal	251	0.88	.31	30,000 (19,000–48,000)	.90	31.8 (31.1–32.5)	.10
Heterozygous‖	98	1.46	.03	27,250 (12,500–48,000)	.96	31.0 (29.9–32.0)	.19
Homozygous or hemizygous**	25	0.48	.07	33,000 (2,800–78,000)	.65	30.2 (27.6–32.8)	.25

* No. of reinfections.
† Incidence rate: median no. of reinfections per child per year (children were observed for >1 year).
‡ Per log-rank test with use of Kaplan-Meier estimates.
§ Median no. of parasites per μL of blood.
# Mean value.
‖ Heterozygous vs. normal (both sexes).
** Homozygous vs. normal and heterozygous.

lower prevalences of *P. falciparum* in individuals with HbAS. Our results show clearly that heterozygous carriers of the sickle cell gene are not protected against infection per se but that infections normally do not lead to high parasite densities; children with HbAS have, on average, almost one-fifth of the parasite density found in those with HbAA.

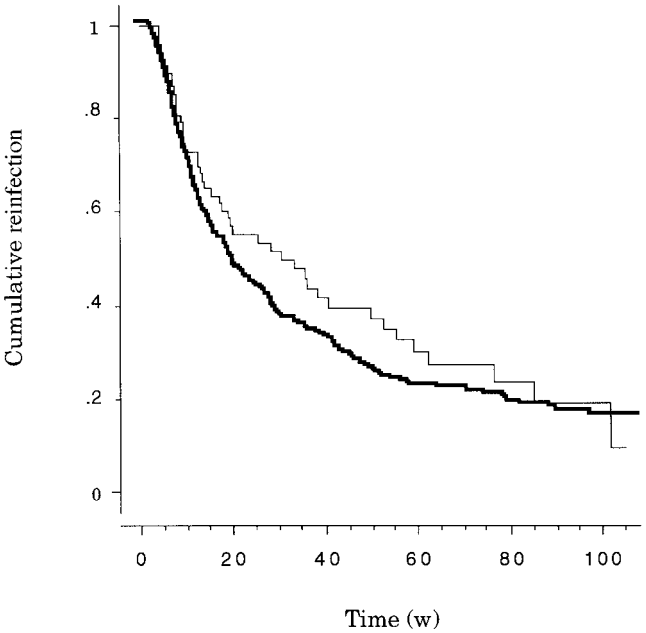

Figure 2. Analysis of not being reinfected over a period of 2 years, for patients with hemoglobin AA (▬) vs. hemoglobin AS (—) (P = .28).

Although a few studies have tried to link the ABO blood group with the incidence of malaria, its protective role against severe malaria has not been adequately studied. Recently, however, a trial in Zimbabwe [20] has shown that patients with blood group A were at a significantly greater risk for severe malaria, with a trend for a protective effect of blood group O. These findings nicely reflect our data, although we cannot confirm a lower hemoglobin value in patients with blood group A. We did, however, find a trend toward higher parasitemia levels in this group.

The mechanisms of this susceptibility remain unclear: on the one hand, given the association of rosetting rate and severe malaria, it could reflect the findings of a higher rosetting rate in group A blood [21]. Among patients in the present study, a higher frequency of rosette-forming isolates was found in the severe-malaria group [6]. On the other hand, an immunologic mechanism is also conceivable, involving, for example, the ability of antibodies to blood group A to react with antigens of *P. falciparum* [22]. In any case, since the allele is maintained at such high frequencies, one would have to postulate that there is a positive selective influence for blood group A that is stronger than the negative effect of malaria in this population.

Strong epidemiological evidence links the distribution of α-thalassemia with the prevalence of malaria in Melanesia [23], and a recent case-control study has shown a significant protection of homozygous α-thalassemia in this area [24]. Given the mild abnormalities of both the heterozygous and

homozygous states, a protective effect against malaria would be expected to be very low [25], and a very large study group would be needed to give sufficient power to detect a difference.

The longitudinal approach of our study made it difficult to admit such a large number of children while ensuring meticulous follow-up. However, while our smaller sample size might lack the power to detect subtle differences concerning polymorphisms occurring at low frequencies, the long-term follow-up should at least lead to the detection of a trend. It is therefore remarkable that the current study was unable to demonstrate even a trend toward a protective effect. Parasite densities in reinfections were higher and incidence densities larger in thalassemic homozygotes, although neither reached statistical significance.

The slightly higher incidence density in homozygous α-thalassemia carriers is not nearly as large as that found in a longitudinal study in Melanesia, where homozygous subjects <5 years of age had over twice the incidence of either *P. falciparum* or *Plasmodium vivax* parasitemia [26]. It was suggested that an increased susceptibility in infancy might result in improved immunity to subsequent severe disease, a concept that is strengthened by in vitro data showing enhanced antigen expression on *P. falciparum*–infected α-thalassemic erythrocytes [27]. The significantly lower hematocrit in homozygous subjects upon reinfection possibly reflects an additive effect of lower hematocrit in healthy α-thalassemic infants [28] and higher levels of parasitemia in these children.

Much controversy has surrounded the question of the protective role of G6PD deficiency in malaria, and several studies have come up with conflicting results [15, 29, 30]. This is partly due to the fact that this disorder has three common X-linked alleles in Africa, which makes analysis and determining the phenotype difficult. Again, the advent of DNA genotyping has eliminated these difficulties, and a large case-control study utilizing DNA genotyping has demonstrated a protective effect of the A− allele against severe malaria in African children [31]. In our study, however, we found no differences at all in the prevalence of any G6PD allele between the mild- and severe-malaria groups. As discussed above for α-thalassemia, the sample size here might be too small to give sufficient power to detect subtle differences attributable to the G6PD deficiency.

The two groups were remarkably similar in the proportion of individuals with G6PD deficiency, which is difficult to reconcile with the findings of the comparable study with Kenyan and Gambian children mentioned above. Furthermore, no protective effect was found by evaluating the reinfection data. Unexpectedly, there was even a significantly higher incidence rate in heterozygous vs. normal individuals, which remains unexplained. Furthermore, we could not confirm the findings of a lower level of parasitemia in males [31] with an A− allele.

In conclusion, in this study we have shown that children with a history of severe disease have a greater risk of reinfection. Severe disease is, to an extent, determined by blood group and the presence of sickle cell hemoglobin, and both α-thalassemia and G6PD deficiency had no protective effect in our study population.

Several thousand years of malaria transmission have helped to shape the human genome, favoring those mutations that afford resistance to malaria. Identifying these alleles could help to detect those children at high risk for malaria as well as broaden our understanding of the mechanisms of disease and protection.

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