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Hemoglobin C and Resistance to Severe Malaria in Ghanaian Children

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Hemoglobin (Hb) C has been reported to protect against severe malaria. It is unclear whether relative resistance affects infection, disease, or both. Its extent may vary between regions and with disease pattern. We conducted a case-control study of children with severe malaria, asymptomatic parasitemic children, and healthy children in Ghana. HbAC did not prevent infection but reduced the odds of developing severe malaria and severe anemia. Protection was stronger with HbAS. The frequencies of HbCC and HbSC decreased, from healthy children to asymptomatic parasitemic children to children with severe malaria. These data support the notion that natural selection of HbC occurs because of the relative resistance it confers against severe malaria but argue against the notion that HbC offers resistance to infection.

Severe *Plasmodium falciparum* malaria continues to be a major cause of morbidity and mortality in sub-Saharan Africa [1]. In regions where malaria is endemic, several hereditary factors are subject to natural selection because of the relative resistance they confer against malaria. This "malaria hypothesis" [2] has been verified for carriers of the sickle-cell trait (hemoglobin [Hb] type AS), who are less likely to experience severe, potentially fatal, malaria [3].

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As with HbS, an amino-acid substitution ($\alpha_2\beta_2\,6^{\text{Glu-Lys}}$) in adult HbA forms the underlying disorder of HbC. HbC occurs in polymorphic frequencies almost exclusively in the northern savannas of western Africa [4–6]. Although HbAC is asymptomatic, HbCC causes mild hemolysis, splenomegaly, or gallstones. Vaso-occlusive illness develops only rarely [7]. The malaria-protective properties of HbC have long been postulated [8]. Recently, relative resistance to severe malaria due to HbC has been reported; however, the findings are partially conflicting. In Mali, HbAC reduced the progression from uncomplicated to severe, mainly cerebral, malaria [4]. This phenomenon was not seen in Burkina Faso, where HbAC and HbCC protected against clinical falciparum malaria per se [5]. Thus far, it remains unclear whether HbC confers relative resistance to infection, to disease, or to both. Moreover, the effect of a protective trait may vary between regions (i.e., between populations and with the predominating disease pattern). In general, severe anemia in young children prevails in areas of high transmission, and cerebral malaria in older patients prevails in areas of lower transmission [1]. The extent to which HbC reduces the risk of severe malaria and of its variable symptoms in areas of different endemicity has yet to be established.

In northern Ghana, where malaria is hyperendemic, we examined whether HbC confers relative resistance to *P. falciparum* infection and/or to severe malaria. In addition, we analyzed the extent to which HbC influences the symptoms and fatality of severe malaria.

Patients and methods. The study was conducted between August and November 2002 (i.e., during the rainy season) in Tamale and its vicinity, Northern Region, Ghana. Tamale is the regional capital of ~300,000 inhabitants but is rural in nature, with hamlets and thatched, mud-wall huts scattered over a vast area. The transmission of malaria in northern Ghana is perennial, and, in the study area, malaria is hyperendemic (authors' unpublished data). The Tamale Teaching Hospital serves as a reference center for the Northern Region. The pediatric ward includes 55 beds but has no facilities for intensive care.

Index patients were 290 children aged 6 months to 9 years who were admitted to the hospital with severe malaria, as defined according to the World Health Organization definition [1, 9]. Informed consent was obtained from the patient's parent(s). The study protocol was reviewed and approved by the ethics committee of the University for Development Studies, Tamale, Ghana, and the institutional guidelines were followed in conducting the present study. For each patient, 2 age- and sex-matched control subjects (1 parasitemic but asymptomatic

Table 1. Distribution of hemoglobin (Hb) types and matched-pair analysis of the risk of severe malaria in Ghanaian children.

Stratum	HbAA	HbAC	HbAS	HbCC	HbSC	HbSS
	Hb type, % (no.) of children					
Healthy control subjects	69.0 (200)	19.0 (55)	8.3 (24)	1.7 (5)	1.7 (5)	0.3 (1)
Parasitemic control subjects	65.9 (191)	24.5 (71)	7.9 (23)	1.0 (3)	0.7 (2)	0 (0)
Patients with severe malaria	82.4 (239)	16.6 (48)	0 (0)	0 (0)	0 (0)	1.0 (3)
Comparisons	OR (95% CI) *P*					
Parasitemic vs. healthy control subjects	Reference	1.32 (0.9–2.0) .2	0.93 (0.4–2.0) 1	0.75 (0.2–3.0) 1	0 (0–2.1) .5	ND
Patients with severe malaria vs. healthy control subjects	Reference	0.76 (0.5–1.2) .27	0.1 (0–0.2) <.0001	0.3 (0–2.1) .5	0.3 (0–0.9) .13	1 (0–99) 1
Patients with severe malaria vs. parasitemic control subjects	Reference	0.53 (0.3–0.8) .005	0.1 (0–0.3) <.0001	0.3 (0–2.1) .5	0.3 (0–2.1) .5	2 (0.2–2.0) 1

NOTE. CI, confidence interval; ND, not determinable; OR, odds ratio.

child and 1 healthy aparasitemic child) were selected from a representative sample of 2107 children from Tamale and the surrounding 6 districts. These children were recruited according to a 2-stage cluster sampling strategy, with probability proportional to population size. Thirty communities and census units were included, and, within each unit, ⩾70 children aged 6 months to 9 years were randomly selected. Venous blood was obtained and stabilized, and DNA was extracted by use of commercial kits (AS1 and QIAmp; Qiagen). Malaria parasites were counted on Giemsa-stained thick blood films per ⩾200 white blood cells, and *P. falciparum* was ascertained by use of polymerase chain reaction (PCR) [10]. Hb types were identified by restriction fragment–length polymorphisms of PCR-generated amplicons [5]. Patients with severe malaria received artesunate (Plasmotrim; Mepha Pharma) for 5 days, at a dose of 5 mg/kg of body weight (double dose on the first day), and supportive care was provided. Parasitemic control children received sulfadoxine-pyrimethamine, if parasitemia was >5000 parasites/μL.

Parasite densities were normalized by $\log_{10}$ transformation, and geometric mean parasite densities (GMPDs) and 95% confidence intervals (CIs) were calculated. Comparison of GMPDs by Hb type within the groups of case patients and parasitemic control subjects, respectively, was done by analysis of variance or Student's *t* test. Matched-pair analysis by McNemar test was performed to evaluate differences in the frequencies of Hb types among case patients and control subjects and to estimate the respective odds ratios (ORs) of severe malaria and of defining symptoms. Exact *P* values were computed by applying the S-Plus (Insightful Software) function *binom.test.* If the total number of discordant pairs was <30, ORs and 95% CIs were estimated by *c*-estimators, because this has been shown to be superior when data are sparse [11]. Two-tailed $P < .05$ was considered to be statistically significant.

Results. Among the 290 index patients with severe malaria (155 girls and 135 boys), the median age was 24 months (range, 6–102 months), the GMPD was 29,512 parasites/μL (95% CI, 21,904–39,763 parasites/μL; range, 15–1,598,000 parasites/μL), the median Hb level was 4.9 g/dL (range, 1.5–13.4 g/dL), and the mean ($\pm$SD) temperature was 38.6°C ($\pm$1.1°C). The following symptoms defining severe malaria were found in the children: severe anemia ($n = 160$ [55%]), prostration ($n = 97$ [33%]), respiratory distress ($n = 66$ [23%]), multiple convulsions ($n = 59$ [20%]), impaired consciousness ($n = 56$ [19%]), jaundice ($n = 34$ [12%]), circulatory collapse ($n = 10$ [3%]), and hemoglobinuria ($n = 8$ [3%]). Hyperparasitemia (defined as >250,000 parasites/μL) was seen in 64 children (22%). Thirty-two of 285 patients who could be monitored died (case-fatality rate, 11.2%).

In both healthy ($P = .97$) and parasitemic control subjects ($P = .68$), the Hb genotype frequencies were in Hardy-Weinberg equilibrium. No significant differences in the distribution of the Hb types were observed between the 2 control groups (table 1). GMPDs in parasitemic children did not differ with Hb type (HbAA, 1879 parasites/μL [95% CI, 1447–2440 parasites/μL]; HbAC, 1486 parasites/μL [95% CI, 911–2424 parasites/μL]; HbAS, 1469 parasites/μL [95% CI, 571–3782 parasites/μL]; HbCC, 6745 parasites/μL [95% CI, 3278–13,882 parasites/μL]; and HbSC, 294 parasites/μL [95% CI, 8–11,132 parasites/μL]; $F = 1.0$; $P = .4$). The highest parasite densities in children with HbCC and HbSC were 11,500 and 1877 parasites/μL, respectively.

In children with severe malaria, HbAS, HbCC, and HbSC were absent, and Hb genotypes deviated from Hardy-Weinberg equilibrium ($P < .0001$). HbAC occurred less frequently in index patients than in parasitemic control subjects, corresponding to a 47% reduction in the risk of developing severe malaria. This figure was 90% for children with HbAS (table 1). The sample size impeded a meaningful analysis for HbCC and HbSC, but both showed significant trends of decreasing frequency, from healthy control subjects to parasitemic control subjects to case patients (HbCC, $\chi^2_{trend} = 4.7$ and $P = .03$; HbSC, $\chi^2_{trend} = 5.4$ and $P = .02$). Among patients with severe malaria, the GMPD was lower in children with HbAC (16,672 parasites/μL [95% CI, 7015–39,626 parasites/μL]) than in children with HbAA (34,594 parasites/μL [95% CI, 25,350–47,209 parasites/μL]; $P = .07$) and

was lowest in children with sickle-cell anemia (HbSS, 785 parasites/μL [95% CI, 78–7920 parasites/μL]; $P = .008$ vs. HbAA).

Finally, we compared Hb genotype frequencies in matched pairs of parasitemic children and children with severe malaria, to examine the role of the Hb variants on symptoms and outcome of severe malaria (table 2). In children with HbAC, the risk of severe anemia was reduced. At borderline statistical significance, this also applied to prostration, multiple convulsions, and fatal outcome. Protection conferred by HbAS was seen for severe anemia and hyperparasitemia. Matched-pair analysis of patients with HbAC or HbAC revealed no significant differences in the respective risks of having symptoms and a fatal outcome but suggested a lower odds of developing severe malaria in children with HbAS (OR, 0.17 [95% CI, 0–0.7]; $P = .06$).

Discussion. In northern Ghana, where malaria is hyperendemic, as in many parts of sub-Saharan Africa [1], severe anemia among young children constitutes the predominant manifestation of severe malaria [9]. Our data provide evidence that HbAC protects against severe malaria in such a setting and against severe anemia in particular. Partially corresponding results have been obtained in regions with a preponderance of cerebral malaria [4, 5]. The interpretation of results should account for statistical limitations involved in multiple comparisons, such as those performed in the present study. On the other hand, when setting that relative resistance to malaria is conferred by HbC and applying 1-tailed analysis, protection against prostration, multiple convulsions, and fatal outcome in children with HbAC becomes statistically significant (data not shown).

In regions where malaria is highly endemic, asymptomatic *P. falciparum* infection is the rule, rather than the exception. In contrast to other investigators [4, 5], we chose aparasitemic healthy children and parasitemic but asymptomatic children as control subjects. It was our intention to examine resistance to infection, on the one hand, and to cover the whole range of disease progression, on the other hand. Therefore, we think that the 2 control groups are appropriate, because aparasitemic children may become infected and because asymptomatic parasitemia may progress to severe malaria. This should be taken into account when estimating the degree of protection against severe malaria conferred by HbAC in the present study (i.e., 47% risk reduction, compared with parasitemic children). This figure was 75% when patients with uncomplicated malaria were used as control subjects in Mali [4] and was 29% in Burkina Faso when children with clinical falciparum malaria were compared with population control subjects [5]. The 95% CIs of the ORs of these studies partially overlap. In a recent study in Tamale, 20.8% of 654 children with uncomplicated malaria had HbAC [6]. When this frequency is compared with that in children with severe malaria (16.6%), a risk reduction of 31% (OR, 0.69; 95% CI, 0.5–1.0) results. Notwithstanding the implicated limitations, this also supports the findings of the present study.

Table 2. Matched-pair analysis of the risk of symptoms and conditions of severe malaria in parasitemic children with hemoglobin (Hb) type AC or HbAS, compared with HbAA.

Condition, hemoglobin type of case patients	No. of pairs, by Hb type of control subjects			OR (95% CI)	*P*
	HbAA	HbAC	HbAS		
Severe anemia					
HbAA	83	35	8	Reference	…
HbAC	19	4	3	0.54 (0.3–0.9)	.04
HbAS	0	0	0	0.20 (0–0.6)	.01
Prostration					
HbAA	50	24	5	Reference	…
HbAC	12	5	0	0.50 (0.2–0.95)	.07
HbAS	0	0	0	0.17 (0–0.7)	.06
Respiratory distress					
HbAA	31	11	5	Reference	…
HbAC	11	3	1	1 (0.4–2.2)	1
HbAS	0	0	0	0.17 (0–0.7)	.06
Multiple convulsions					
HbAA	36	10	5	Reference	…
HbAC	3	2	2	0.42 (0.1–1.0)	.09
HbAS	0	0	0	0.17 (0–0.7)	.06
Impaired consciousness					
HbAA	27	12	5	Reference	…
HbAC	5	1	2	0.50 (0.2–1.2)	.14
HbAS	0	0	0	0.17 (0–0.7)	.06
Jaundice					
HbAA	15	10	0	Reference	…
HbAC	6	1	1	0.67 (0.2–1.6)	.45
HbAS	0	0	0	ND	ND
Circulatory collapse					
HbAA	5	2	2	Reference	…
HbAC	0	0	0	0.33 (0–2.1)	.50
HbAS	0	0	0	0.33 (0–2.1)	.50
Hemoglobinuria					
HbAA	7	0	0	Reference	…
HbAC	1	0	0	2 (0.2–2.0)	1
HbAS	0	0	0	ND	ND
Hyperparasitemia					
HbAA	40	9	6	Reference	…
HbAC	4	3	1	0.55 (0.1–1.4)	.27
HbAS	0	0	0	0.14 (0–0.6)	.03
Fatal outcome					
HbAA	13	10	1	Reference	…
HbAC	3	1	1	0.42 (0.1–1.0)	.09
HbAS	0	0	0	0.50 (0–6.5)	1

NOTE. No significant associations with severe malaria symptoms were seen for the remaining Hb genotypes (HbCC, HbSS, and HbSC). CI, confidence interval; ND, not determinable; OR, odds ratio.

On the basis of the present data, we cannot comment on protection against uncomplicated malaria afforded by HbAC. However, in a longitudinal study in Mali, a reduced rate of mild malaria episodes and lower parasite densities were observed in children with HbAC [12]. HbCC and HbSC were absent in patients with severe malaria and were less frequent in parasitemic

than in healthy control subjects. Although the small number of subjects impaired separate analysis, this result points to protection from malaria, which is in accordance with results of other researchers [5]. The question remains as to why HbC is localized to a relatively small region and has not spread further. One explanation could be that the origin of the β^C mutation is recent. Modiano et al. [5] suggested that similar protective effects of HbC and HbS, together with the disadvantage of the HbSS homozygote, would lead in the long term to a replacement of HbS by HbC in western Africa. However, the present study shows that protection conferred by HbAS (90%) is stronger than that conferred by HbAC (47%). In evolutionary terms, the effect of this advantage might not be offset by the disadvantage of the homozygous state.

The mechanism of relative resistance in HbC remains obscure. On the basis of our data, resistance to infection itself is unlikely. In fact, HbAC tended to be more frequent in parasitemic than in healthy control subjects. Reduced parasite densities reported in children with HbAC [12] could not be confirmed in our control subjects or in other studies [4, 6]. In vitro, HbAC erythrocytes support the growth of *P. falciparum* [13], whereas the replication rate is substantially reduced in HbCC red blood cells [13, 14]. This phenomenon could explain the relatively low maximum parasite density in control subjects with HbCC and HbSC. A recent in vitro study demonstrated that reduced parasite multiplication in HbCC erythrocytes is not due to a lower invasion rate. Rather, parasites disintegrated in about one-half of these cells. Moreover, knob formation (i.e., the expression of parasitic neoantigen on the erythrocyte membrane) was found to be modified in HbCC erythrocytes. Because knobs are involved in the cytoadherence of infected erythrocytes and contain proteins of antigenic importance, the individual course of malaria in patients with HbCC may be influenced by reduced parasite replication, the modified expression of surface antigens, and aberrant cytoadherence [14]. In addition, increased phagocytosis of parasitized red blood cells could add to the mechanism of protection in HbCC, as has been suggested for α-thalassaemia [15] and has been observed in glucose-6-phosphate dehydrogenase deficiency [16]. In vitro data on HbAC erythrocytes in this regard are lacking, but changes similar to those seen in HbCC cells and related consequences might occur, to a lesser extent, in the heterozygous trait as well. This could result in enhanced development of clinical immunity, as has been proposed for other erythrocyte variants [15]. Another mechanism could be the selection of relatively avirulent *P. falciparum* strains. However, the prevalence and number of distinct parasite clones in children with HbAC show no substantial deviation from those in individuals with HbAA [6].

We have provided evidence for the natural selection of HbC in a population with a high burden of malaria, particularly severe malarial anemia. Thus far, studying host resistance to malaria has not resulted in new therapeutic principles or antimalarial interventions. Still, these findings may help to anticipate epidemiological and clinical consequences once such measures are introduced in areas where HbC is common.

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