Original Contribution

Sickle Cell Trait Protects Against *Plasmodium falciparum* Infection

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Although sickle cell trait protects against severe disease due to *Plasmodium falciparum*, it has not been clear whether sickle trait also protects against asymptomatic infection (parasitemia). To address this question, the authors identified 171 persistently smear-negative children and 450 asymptomatic persistently smear-positive children in Bancoumana, Mali (June 1996 to June 1998). They then followed both groups for 2 years using a cohort-based strategy. Among the 171 children with persistently negative smears, the median time for conversion to smear-positive was longer for children with sickle trait than for children without (274 vs. 108 days, \( P < 0.001 \); Cox hazard ratio = 0.56, 95% confidence interval: 0.33, 0.96; \( P = 0.036 \)). Similar differences were found in the median times to reinfection after spontaneous clearance without treatment (365 days vs. 184 days; \( P = 0.01 \)). Alternatively, among the 450 asymptomatic children with persistently positive smears, the median time for conversion to smear-negative (spontaneous clearance) was shorter for children with sickle trait than for children without (190 vs. 365 days; \( P = 0.02 \)). These protective effects of sickle trait against asymptomatic *P. falciparum* infection under conditions of natural transmission were demonstrable using a cohort-based approach but not when the same data were examined using a cross-sectional approach.

asymptomatic infection; cohort- and cross-sectional-based studies and analyses; *Plasmodium falciparum*; sickle cell trait

Abbreviations: Hb AA, hemoglobin A homozygote; Hb AS, hemoglobin AS heterozygote.
treatment to clear asexual *P. falciparum* parasites from the blood.

**Underlying hypothesis (biologic concept or framework)**

The hypothesis underlying these studies was that Hb AS protected against asymptomatic *P. falciparum* infection (and also facilitated the spontaneous clearance of infection in the absence of drug treatment) in the same way that it protected against symptomatic *P. falciparum* infection. To test for factors such as Hb AS that could prevent (or reduce the frequency of) asymptomatic *P. falciparum* infection and potentially relevant confounders, we performed a prospective, cohort-based study in the village of Bancoumana in Mali. The rationale for using a village-based study was to focus on subjects with asymptomatic infection. The rationale for using a cohort-based protocol was to increase the power of the study by permitting subjects to serve as their own controls, because conflicting and ambiguous results have been obtained in the past using cross-sectional study design and evaluation strategies.

**MATERIALS AND METHODS**

**Study site and population**

These studies were performed in Bancoumana, a rural village with a population of 8,000 located 60 km southwest of Bamako, the capital city of Mali, and 5 km from the west bank of the Niger River. The village is 1.5 × 1.8 km, is situated at 8° 20' longitude west and 12° 20' latitude north, and has a mean altitude of 355 m (maximum = 481, minimum = 229). Bancoumana belongs to the administrative district of Siby in the Guinée Savanna region of Mali. Malaria transmission is hyperendemic and seasonal (June–November).

Malinké are the most common ethnic group (84%), followed by Fulani (8%) and others (Bambara, Somono, and Bozo). The Malinké are typically farmers, whereas the Fulani are herders. The population is 95% Muslim and 5% Animist.

Bancoumana established a dispensary in 1958 and a maternity ward in 1965. In 1993, the dispensary became a Community Health Center (Centre de Santé Communautaire, Benso). A physician hired by the health center provides medical care for the community. A board of directors comprising the physician and representatives of the village manages the resources available (infrastructure, essential drugs, and consultation fees) and sets priorities for the Community Health Center. Two pediatricians and a team of investigators from the Mali-Tulane Tropical Medicine Research Center assisted the Center in providing free diagnosis, treatment, and follow-up care for malaria in children who were residents of Bancoumana.

**Study sample and informed consent**

The study sample consisted of 621 children, each of whose parents or guardians had already provided informed consent for their participation in the village-wide, prospective cohort study: 171 persistently smear-negative children and 450 persistently smear-positive children. (Definitions of terms are provided in Appendix 1.) After these children had been identified by review of the integrated parasitologic/demographic database in June 1997, their parents and guardians were interviewed a second time to obtain informed consent for participation in this study. Before presentation to the chief, elders, women’s council, and the assembled parents of the village, the study protocol was reviewed and approved by institutional review boards at the University of Bamako in Mali and Tulane University in New Orleans, Louisiana.

**Study protocol for the entire village-wide cohort**

From June 1996 to June 1998, we followed the village-wide cohort of 2,000 children with thick smears 5 times each year (June, August, and October during the rainy season; December and February or March during the dry season). During those surveys, finger-stick blood samples were obtained from the 2,000 children in the village-wide cohort for microscopy (thick smears) and molecular studies (polymerase chain reaction using parasite DNA extracted from filter paper blots). In addition, children and their parents or guardians were asked about symptoms and signs consistent with malaria to identify and treat persons with symptomatic infections. These data were then linked to demographic, socioeconomic, behavioral, entomologic, and geographic information system data in a central computerized database and used to test for host factors that might affect the frequency of infection (*P. falciparum* parasitemia).

**Study protocol for persistently negative and persistently positive subcohorts**

Children whose parents or guardians provided informed consent were examined 5 times each year in the village-wide cohort as previously described (Figure 1). In addition, the 621 children for whom informed consent was obtained for participation in the present study were also interviewed about potential risk (and protective) factors for *P. falciparum* infection, including maternal educational level, antimalarial drug and bed net use, and family financial status. During February 1998, we obtained a single 5-mL venous blood specimen from each of the 621 children in the persistently negative and persistently positive subcohorts to identify hemoglobin type (by starch gel electrophoresis), ABO and MN blood group status (by agglutination), and in selected instances, antibody status based on indirect fluorescent antibody testing for antibodies to asexual stage *P. falciparum* parasites (21). Smear-positive children who developed symptoms of malaria were treated at the Community Health Center and then excluded from the database for a period of 4 weeks after treatment (treatment was oral chloroquine for uncomplicated malaria and parenteral quinine for severe malaria). Clearance of parasitemia after antimarial treatment was not counted as a spontaneous clearance event.
Microscopy

Thick smears were stained with Giemsa and examined under oil immersion magnification (×1,000) to estimate the number of asexual *P. falciparum* parasites per 300 leukocytes. Fields containing 1,000 or more leukocytes were examined for negative specimens. The number of asexual *P. falciparum* parasites per μL of blood was estimated by multiplying the number of asexual parasites per 300 leukocytes by 25, based on an average leukocyte count of 7,500 per μL (22).

Antimalarial treatment and the frequency of severe disease

On the basis of Ministry of Health guidelines, antimalarial treatment was provided for persons with symptomatic *P. falciparum* infections but not for persons with asymptomatic infections. With this strategy, severe disease (cerebral malaria) declined progressively over the first 4 years of this project, from 50–65 cases per year to 2–3 cases per year. As a result, it was necessary to move studies of cerebral malaria to Bamako, which also draws on other surrounding villages and on the capital city of Bamako, with its population of 2 million.

Statistical analyses

Parasite counts were transformed by adding 10 to their original values and taking the logarithm (base 10) to normalize the distribution (23); a value of 1 thus corresponded to a count of 0 and 5 to 100,000 parasites per μL. Geometric mean parasite densities were calculated by back transformation of the means of these log-transformed values. Means of the transformed parasite counts were then compared for the variables of interest using the Student’s t test or analysis of variance. Entomologic inoculation rates were transformed by adding 1 to their original values and taking the natural logarithm. Bivariate and multivariate analyses were performed using logistic regression and multinomial logistic regression (23–25) to examine the relation between *P. falciparum* infection and potential risk factors (e.g., age, sex, hemoglobin, antimalarial drug use, and entomologic inoculation rate).

RESULTS

Baseline information on the study sample

Among the 5 characteristics examined in relation to parasitemia (age, sex, ethnic group, ABO blood group, and hemoglobin type), 2 diverged from the expected: age and ethnic group (Table 1). With respect to age, persistently positive smears were more common among children 5–9 years of age than among children 6 months to 4 years of age (*P* = 0.001). With respect to ethnic group, persistently positive smears were more common among Malinké than among Bambara or Fulani children (397 of 524 vs. 44 of 80; *P* = 0.001).

Odds ratios for a multivariate logistic model of persistent parasitemia

Using the Hosmer Lemeshow model-building strategy (23), we identified 3 risk factors for inclusion in a multiple logistic regression model: age (children 5–9 years of age were smear-positive more frequently, *P* < 0.001), lack of antimalarial drug use (which was related to persistent smear positivity, *P* = 0.01), and an enlarged (palpable) spleen (*P* < 0.001; Web Table 1, available at http://aje.oxfordjournals.org/). In addition to age, factors related to persistent smear positivity in the final logistic regression model (23–25) included an enlarged (palpable) spleen (more significant risk factor for children 6 months to 4 years of age than for children 5–9 years of age), lack of antimalarial drug use (*P* = 0.02), and a thatch (straw) roof (*P* = 0.001) (Table 2 and Web Tables 1 and 2). Because thatch roofs are inexpensive, other variables were examined...
to determine whether this association simply reflected socioeconomic status. Socioeconomic variables potentially related to environmental exposure (home and land ownership) were associated with an increased risk of parasitemia (odds ratios = 4.15–5.88 and 2.56–6.46, respectively). Conversely, variables unrelated to environmental exposure (owning a radio or television) were not (odds ratios = 0.39–0.55 and 0.19–0.34, respectively).

Cox hazard ratios for individual risk factors

Risk factors for persistent parasitemia identified from their Cox hazard ratios (24) included age from 5 to 9 years (P = 0.002), splenic enlargement (P = 0.016), and hemoglobin A homozygote (Hb AA) (P = 0.036 relative to Hb AS) (Table 3).

Time to initial infection among smear-negative children

Among the 171 children with persistently negative smears in 3 (or 2) of the surveys during the 1996 transmission season, children with Hb AS remained smear-negative longer than did children with Hb AA (median times of 274 vs. 108 days; Kaplan Meier plot, Figure 2). On the basis of the nonparametric log rank test, these differences were significant (P < 0.001). These differences were not due to clustering of Hb AS and Hb AA participants in areas of the village with less or more intense transmission (Figure 3A) because both Hb AA and Hb AS were spread across the entire village, as were the different ABO blood groups (Figure 3B). In contrast, there was clustering of persistently infected children near the river and irrigated rice fields and of persistently uninfected children in other areas of the village (Figure 3C). This result was not influenced by differences in Hb types between children with Hb AA versus those with Hb AS because similar patterns were observed when the results for all children (Web Figure 1) were compared with those for children with Hb AS versus those with Hb AA (Figure 2).

Reinfection after spontaneous clearance among smear-negative children

Likewise, children with Hb AS remained smear-negative longer than did children with Hb AA (median times of 365 vs. 184 days, respectively; P = 0.01; data not shown) after
Spontaneous clearance among smear-negative children

Among the 171 children who were persistently smear-negative during 1996, all but 2 (both Hb AS) became infected subsequently; 58 of the 169 cleared their infections without antimalarial treatment. In addition, the duration of infection (smear positivity) was greater for children with Hb AA than for children with Hb AS who had been reinfected after the spontaneous clearance of previous P. falciparum infection (Web Figure 2).

Sickle-cell trait, parasite density, and the risk of disease

Among children with positive thick smears, geometric mean parasite densities were higher for children with Hb AA than for children with Hb AS (275 vs. 131 parasites per μL, respectively; P = 0.002). Consistent with that observation, children with Hb AS had a lower incidence of disease, that is, they were treated for malaria less frequently at the Bancoumana Community Health Center (controlling for age, odds ratio = 0.68, 95% confidence interval: 0.54, 0.87; P = 0.002).

Effect of bed net use

On the basis of univariate analysis, bed net use was associated with a lower risk of infection during both years of the study (odds ratios = 0.52 and 0.34, 95% confidence intervals: 0.31–0.87 and 0.15–0.76; P values of 0.012 and 0.009, respectively). However, bed net use was not an independent predictive risk/protective factor in the multivariate analysis (Web Table 1).

Cross-sectional versus cohort (prospective) study design

To test the influences of study design and evaluation strategies, we examined the association between sickle-cell trait and P. falciparum infection using both cross-sectional and cohort-based evaluation strategies to study the same dataset. Using a cohort-based approach (log-rank test) (23), significant differences between Hb AS and Hb AA children first appeared in March 1997 and continued through to the conclusion of the study in June 1998. In contrast, when the same data were examined using a cross-sectional approach, the differences between Hb AS and Hb AA children never achieved significance during the 24-month observation period, from June 1996 to June 1998 (Table 3).

DISCUSSION

Threshold (sensitivity) of microscopy

One potential confounder in these studies was the possibility of false-negative thick smears with asymptomatic P. falciparum infection. However, 2 lines of evidence suggest that this was not a problem. First, if false-negative thick smears were common in asymptomatic children, those children should have had few positive smears. In fact, among the 2,000 children in the cohort, 450 asymptomatic children had persistently positive smears. Second, because of concern about this issue, we estimated the frequency of false-negative thick smears by polymerase chain reaction using 3 sets of allotype-specific primers for the trimorphic block 2 region of the merozoite surface protein 1 (msp1) gene (26). None of the 200 samples amplified from specimens with negative thick smears yielded positive results by polymerase chain reaction. In contrast, more than 98% of samples from children with positive thick smears yielded amplicons on agarose gel electrophoresis (O.A. Koita, University of Bamako, unpublished observations). These 2 lines of evidence suggest that the frequency of false-negative thick smears was low, and thus that this issue did not confound either the analysis or the interpretation of the data.

Potential bias from children recruited or studies performed after June 1996

To determine whether children enrolled after June 1996 (i.e., in August 1996) were a potential source of bias, we compared the Kaplan-Meier plots for conversion from persistently negative to positive (Figure 2) and for conversion from persistently positive to negative (Figure 4). Because no differences were found, we concluded that there was no confounding and have therefore presented the data for both groups together. Because venipunctures for Hb typing and
ABO blood grouping were performed more than 6 months after the time of initial enrollment, a number of children were missed or chose not to participate in this part of the study. However, the high level of participation (740 of 851; 87%) and the similarity of the data obtained to information from other sites in Mali suggest that the Hb types and ABO blood groups found were similar to those in the general population.

Figure 3. Clustering of hemoglobin types, blood groups, and persistently infected and persistently uninfected children, Bancoumana, Mali, 1996–1998. In each of these 3 panels, roads are indicated by solid lines, depressions by light gray, breeding sites by dark gray, and the rice field in the lower right by a mottled, patchy gray fill. A) Children with hemoglobin AS (filled squares) and hemoglobin AA (open squares) were distributed across the village of Bancoumana without evidence of clustering. B) Likewise, children with blood groups A (open stars), B (filled stars), and O (open circles) were distributed across the entire village without evidence of clustering. C) In contrast, persistently infected children (open triangles) were clustered on the southern side of the village near the river and the irrigated rice field. Conversely, persistently uninfected children (filled triangles) were clustered in areas of the village away from the river and the irrigated rice field.
Age and ethnic differences

Because children 6 months to 4 years of age were treated for symptomatic infection more frequently at the Community Health Center than were children 5 to 9 years of age (P < 0.01), their infections may have been cleared more frequently with antimalarial drugs, secondarily producing a greater frequency of persistently smear-negative children who were 6 months to 4 years of age. In addition, the ethnic differences in the frequency of \textit{P. falciparum} infection reported from Burkina Faso (27, 28) were consistent with the lower infection rates observed by others in Fulani and Bambara children (Table 1). In those reports, lower rates of infection were observed among the Fulani. As in these studies, there were no differences in the frequency of Hb AS among Malinké versus Fulani (or Bambara) children. Although the numbers of Fulani and Bambara
children in the present study were small, the results were consistent with those obtained by Modiano et al. (27, 28), who studied larger numbers of subjects. Both these results and those of Modiano et al. suggest that Fulani and Bambara children share additional genetic or environmental determinants (other than Hb AS) that protect them from *P. falciparum* infection.

**Protection from symptomatic infection and high parasite densities**

The protection against symptomatic infection (uncomplicated malaria) provided by sickle-cell trait that we observed is consistent with the reports of many investigators (14–18, 29–31), beginning with Beet, Allison, and their colleagues more than 50 years ago (4–6). This result, like the lower geometric mean parasite densities associated with Hb AS (14–18, 28, 31), provides a positive internal control because it is consistent with the results of other investigators.

**Final logistic model of parasitemia and Cox hazard ratios**

The association between splenomegaly and *P. falciparum* infection (Tables 1 and 2) presumably reflects the production of splenomegaly as a result of persistent parasitemia (27, 32) rather than vice versa, although an association does not establish a cause-and-effect relation. The
association with thatch roofs (Table 1) presumably reflects the larger numbers of Anopheles gambiae in houses with thatch roofs (vs. those with tin roofs), with secondary increases in the biting rates and entomologic inoculation rates, and is consistent with other reports (33). The protective effect of Hb AS (Table 3) is consistent with the results in Figures 2 and 4.

Plots of infection, reinfection, and spontaneous clearance

Taken together with the Cox hazard ratios (Table 3), the longer times to initial infection (Figure 2) and reinfection (data not shown) and the more rapid clearance times observed in children with Hb AS (Figure 4) establish that sickle-cell trait reduces the frequency of asymptomatic P. falciparum infection and increases the clearance of asymptomatic P. falciparum infection. In contrast to a number of previous studies, the data provided here were restricted to children with asymptomatic infections and excluded the effects of treatment.

Effect of study design on statistical evaluation

To evaluate the effect of study design on the ability to detect an impact of Hb AS on P. falciparum infection, the results for the same data set (Kaplan-Meier plot in Figure 2) were compared using both cohort-based (log rank test) and cross-sectional (χ²) statistical methods. The results (Table 4) demonstrate the power of the cohort-based study strategy. They suggest that the cross-sectional study strategy that has been used to examine this issue previously is one of the reasons it has been difficult to demonstrate the protective effect of Hb AS against asymptomatic P. falciparum infection.

Biologic implications

The pathophysiologic (mechanistic) explanations provided previously for the protective effect of Hb AS against severe disease are similarly applicable to the protective effects of Hb AS against asymptomatic P. falciparum infection (inhibition of parasite growth as a result of sickling in the peripheral circulation (34) where red cells with P. falciparum parasites are sequestered as parasites mature and ligands (e.g., P. falciparum erythrocyte membrane protein 1) for cytoadherence to endothelial cell receptors are expressed on the red cell surface) (35). Thus, the results reported here are consistent with what is known about the pathophysiology of sickle-cell trait (36, 37) and the cytoadherence of P. falciparum-infected red blood cells (35). They suggest that the prevalence of sickle-cell trait in a population may reduce not only the incidence of severe disease, as shown previously (16, 38, 39), but it...
may also decrease the intensity of transmission by reducing the prevalence of parasitemia in the asymptomatic human population.

Conclusions

These results indicate that sickle-cell trait (Hb AS) protects against asymptomatic *P. falciparum* infection in children who are initially uninfected (smear-negative) and in children who have cleared their infections (either with (20) or without antimalarial drugs). They likewise indicate that persons with sickle-cell trait clear asymptomatic *P. falciparum* infection faster than do controls (Hb AA) in the absence of antimalarial treatment. Although the protective effects of Hb AS against disease due to *P. falciparum* have been known for more than 50 years, it has not been clear whether Hb AS also protected against asymptomatic infection. The results reported here establish that Hb AS protects against asymptomatic *P. falciparum* infection under the conditions of natural transmission.

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Conflict of interest: none declared.

REFERENCES

APPENDIX 1

Definitions of Terms

Asymptomatic infection: The presence of asexual Plasmodium falciparum parasites on thick smear in the absence of fever, chills, malaise, or other symptoms or signs of malaria.

Symptomatic infection: The presence of asexual P. falciparum parasites on thick smear with fever, chills, malaise, headache, myalgias, or other symptoms or signs of malaria.

Spontaneous clearance (spontaneous recovery from P. falciparum infection): Clearance of asexual P. falciparum parasites from the blood (conversion from smear-positive to smear-negative) in the absence of antimalarial treatment. Although identified most commonly by examining smears from sequential cross-sectional studies within the village-wide cohort, conversions from smear-positive to smear-negative were also identified by examining smears from subcohort studies within the village-wide cohort and smears from symptomatic persons attending the Bancoumana Community Health Center.

Mild (uncomplicated) malaria: Fever, chills, malaise, myalgias, headache, or other symptoms or signs of malaria with a thick smear positive for asexual P. falciparum parasites. Exclusions for severe or complicated malaria included coma, seizures, pulmonary edema, hypoglycemia (glucose <40 mg per dL), and severe anemia (Hb <5 g per dL).

Persistently negative (smear-negative) children (n = 171): children who had negative smears during each of the 3 surveys performed in the transmission season 1996 (June, August, and October) or who were recruited in July and had negative smears during each of the subsequent 2 surveys (August and October).

Persistently positive (smear-positive) children (n = 450): children who had positive smears for P. falciparum during each of 3 surveys in the 1996 transmission season (June, August, and October) or children recruited in July and had positive smears during each of the 2 subsequent surveys (August and October).