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Longitudinal study of *Plasmodium falciparum* infection and immune responses in infants with or without the sickle cell trait

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Background	Individuals may be homozygous (SS) or heterozygous (AS) sickle cell gene carriers or have normal adult haemoglobin (AA). Haemoglobin S could have a protective role against malaria but evidence is sparse and the operating mechanisms are poorly known.
Methods	We followed two cohorts of children. The first was enrolled at birth (156 newborn babies) and the second at 24–36 months old (84 children). Both cohorts were followed for 30 months; monthly for parasitological data and half yearly for immunological data.
Results	In the first cohort, 22%, and in the second 13% of children were AS. Whatever their age parasite prevalence rates were similar in AA and AS individuals. Mean parasite densities increased less rapidly with age in AS than in AA children, and were significantly lower in AS than in AA children >48 months old. The AA children tended to be more often admitted to hospital than AS children (22% versus 11%, NS). Both anti-*Plasmodium falciparum* and anti-Pf155/RESA antibody rates increased more rapidly in AA than in AS children. Conversely, the prevalence rate of cellular responders to the Pf155/RESA antigen was similar in AA and AS children during the first 2 years of life, then it was higher in AS than in AA children.
Conclusions	Sickle cell trait related antimalarial protection varies with age. The role of the modifications of the specific immune response to *P. falciparum* in explaining the protection of AS children against malaria is discussed.

Keywords	*Plasmodium falciparum*, malaria, sickle cell trait, children, immunity, epidemiology
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In areas where malaria is endemic, the highest death toll is among young children who have not yet developed protective immune mechanisms against the parasite. Repetition of infection allows the build-up of incomplete and unsteady immunity (termed premunition) which does not prevent infection, but controls the parasite density keeping it under the pathogenic threshold. Besides acquired immunity, some individuals appear to be naturally provided with biological advantages which partially impede parasite growth. For example, the HLA BW 53 class 1 antigen was reported to grant some protection against cerebral malaria.[1,2] Abnormal haemoglobins (S, C, E) would lead to a balanced polymorphism within malaria endemic areas as they are associated both with a reduction in the number of homozygous individuals whose life expectancy is limited, and with an extension of the duration of life of heterozygous individuals (as well as a reduction of the risk of death before 5 years of age) due to inborn protection against malaria.[3] Nevertheless, evidence of such a genetic process is still lacking, the operating mechanisms are poorly known, and the existence of such a selective advantage still remains disputed. The most studied structural modification within haemoglobin concerns haemoglobin S. Allison[4] first proposed its protective quality against malaria. It was later confirmed that, during the first years of life, the malaria-related morbidity rate was significantly lower in heterozygous sickle cell gene carriers (AS) than in subjects with normal adult haemoglobin (AA), although parasite prevalence rates were similar in both groups.[5–7] Various mechanisms have been evoked, including the falciformation of parasitized red blood cells leading to increased phagocytosis of these cells, polymerized haemoglobin S being an unsuitable substrate for *Plasmodium*

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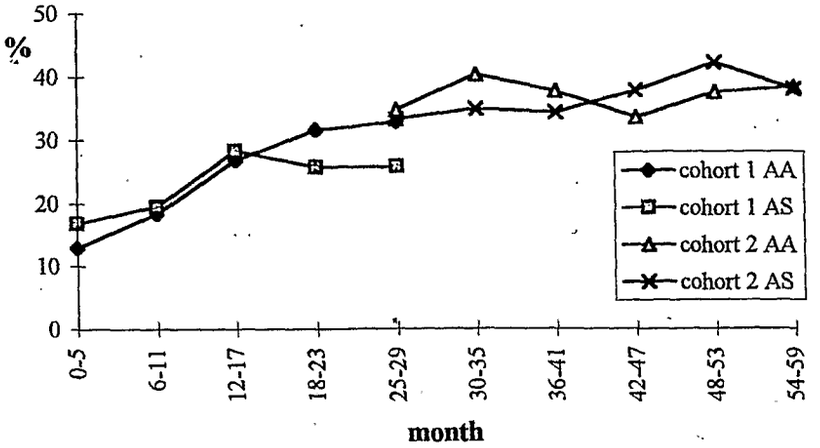

Figure 1 Six monthly evolution of parasite prevalence in children of the two cohorts presenting with AA: cohort 1 (—◆—), cohort 2 (—△—) or AS: cohort 1 (—■—), cohort 2 (—✕—) haemoglobin

falciparum proteases, alterations of the erythrocyte membrane and slower disappearance of haemoglobin F.[8,9]

Alternatively, it was suggested that the protective effect of haemoglobin S was not located at the erythrocyte level but was linked to some modulation of the immune response against malaria,[10] including an earlier acquisition of protective immunity by AS, as compared to AA children.[11] All these assumptions remain controversial and we compared AA and AS children at different stages of infancy in order to characterize the relation between malaria and sickle cell trait.

Population and Methods

Area, climate and population

The study was conducted from January 1993 to December 1995 in Ebolowa, a town of 35 000 inhabitants in South Cameroon, 160 km south of Yaoundé, the capital. The region is characterized by an equatorial climate. Rain falls all year round (1700 mm) with two peaks, from September to December and from April to June, allowing the persistence of anopheline breeding sites all year round, and perennial transmission of malaria parasites. The natural rainforest surrounding the town has been altered by coffee and cacao growing and other agricultural activities. The main ethnic groups are the Bulu people (a subgroup of the Bantu) and groups from Western (Bamileke) and Central (Eton, Ewondo, Bassa) Cameroon.

Health services are provided through the government provincial hospital (Ekombitie hospital), a mission hospital (EPC Enongal hospital), and government or church health centres. Recourse to health services is usual but self-medication is frequent too. Drugs circulate widely, being sold through drugstores or at the market place. Oral chloroquine is currently the most widely used treatment for uncomplicated malaria, whereas intramuscular or intravenous quinine is used for severe malaria.

Clinical, parasitological and biological monitoring

The first cohort consisted of children enrolled at birth, and the second of children from the same family but aged 24–36 months at enrolment. For 6 months, all pregnant women living in or within 10 km of Ebolowa and delivering at one of the two maternity facilities in Ebolowa were invited to enrol their children. New-born babies were enrolled at birth in cohort 1. At the same time, an older sister or brother, if any and if aged 24–36 months, was enrolled in cohort 2. Children from both cohorts were followed-up for 30 (for the last enrolled) to 36 (for the first enrolled) months. Informed consent was given by two parents.

At inclusion in cohort 1, a questionnaire was filled in for each child with information related to the pregnancy history and the new-born baby. Blood samples were collected from the cord and the mother. Blood smears were done on the cord and the mother's peripheral and placental bloods. Weekly, each child from both cohorts was visited at home and their temperatures recorded. In case of fever (axillary temperature >37.5°C), a thick blood smear was done. Each month, a blood smear was done to detect symptomless parasite infections. When a child presented to the health centre seeking care between our visits, information was collected from the medical report. A cross-sectional survey was conducted in each family during the follow-up to collect information related to the children's environment and prophylactic use of drugs, and the socioeconomic status of the family.

Every half-yearly period, a blood specimen was obtained for haematological and immunological measurements and once for haemoglobin genotype assessment.

Children as patients

When a child had fever, a thick blood smear was made and rapidly read in the hospital's laboratory facilities. When malaria parasites were present, the child was given antimalarial treatment. During the first year, chloroquine was given (25 mg/kg over 3 days) but in the course of the second year of the follow-up, amodiaquine (25 mg/kg over 3 days) was used for treatment because of the occurrence of therapeutic failures with chloroquine. Children presenting with severe malaria and/or a parasite density >100 000 parasites per µl of blood were referred to the hospital staff. During the study, all care and drugs related to malaria were free for all participants. Parents were advised of

Table 1 Socio-demographic characteristics of AA and AS children (data are expressed as per cents of the total)

		AA children (n = 195)	AS children (n = 45)	*P*
Sex	Male	81	19	NS
	Female	81	19	
Ethnic group	Bulu	74	26	0.005
	Bamileke	98	2	
	Centre	86	14	
	Others	81	19	

the results of all tests carried out by the team, and were given therapeutic advice when necessary. Once a week, parents could meet a physician to get information on and explanation about the study.

Laboratory methods

All thick blood smears were Giemsa-stained and examined against 200 leukocytes if positive, or against 400 leukocytes prior to being declared negative. Parasite densities were recorded as the number of parasites/µl of blood, assuming an average leukocyte count of 8000/µl, thus the threshold of sensitivity was 20 parasites/µl. In the OCEAC laboratory in Yaoundé, routine quality control ensured the re-examination of a random sample of blood smears, which were examined without being aware of the previously recorded result.

Haemoglobin genotype was determined by electrophoresis on cellulose acetate gel with alkaline buffer.

The humoral and cellular immune response of the children was investigated every 6 months. The level of plasma antibodies directed against *P. falciparum* and the Pf155/RESA antigen (a major antigen from asexual blood stages of *P. falciparum*) were assessed by ELISA and by erythrocyte membrane immunofluorescence (EMIF), respectively, as previously described.[12,13] The *in vitro* lymphocyte proliferation in response to leucoagglutinin (10 µg/ml), tuberculin purified protein derivative (PPD)(12.5 µg/ml), and purified Pf155/RESA protein (5 µg/ml) was measured by ^{3}H-thymidine incorporation, as described. All these tests were performed in the OCEAC immunological laboratory in Yaoundé.

Statistical analysis

Due to cultural and commercial reasons, Ebolowa inhabitants travel frequently between the town and their villages of origin. During the course of the follow-up, a mean of 60% of the children were seen during each monthly visit. Children away for more than 6 months were considered as lost to follow-up. The frequency and the regularity of the follow-up procedure, as assessed by the mean follow-up duration and the mean age of the children being followed-up, were compared by age group between AA and AS children by ANOVA. Children were tested for immunological survey every 6 months (±1 month). All the blood-smear results from the half-yearly period were taken into account for the analysis of the relation between sickle cell trait and malaria. Pearson's χ^2 test was used for contingency table data. Geometric mean parasitaemias by age group were calculated after log transformation and were compared by analysis of variance if variances were homogeneous or by a non-parametric test (Kruskall Wallis) if not. Statistical analysis was performed using EPI-INFO 5.0 (Centers for Disease Control and Prevention, Atlanta, USA) and EGRET (Serc, Seattle, USA) computer softwares. *P*-value <0.05 was considered statistically significant.

Results

From January to June 1993, 277 mothers were contacted in the two maternity hospitals in Ebolowa. Twenty-three families likely to leave Ebolowa before the end of the follow-up were not included. Ten families refused follow-up. A total of 244 new-born babies were included at birth in cohort 1 and 126 children 24–36 months old in cohort 2. Two and a half years later, 36% of children from cohort 1 and 33% of children from cohort 2 were lost to follow-up. Seven children died.

In the first cohort, 156 infants including 34 (22%) AS children were followed-up between the ages of 0 and 30 months. Eighty-four children, including 11 AS children (13%) were included in the second cohort. Table 1 shows the distribution by sex and ethnic group. The distribution of the AS individuals by ethnic group was very heterogeneous ($P = 0.005$), the lowest value prevailing among the Bamilekes (2%) and the highest among the Bulus (26%). In African cities, families tend to line in areas close to those coming from a similar area. A total of four infants were SS, all belonging to the first cohort, and were not taken into consideration in further analysis.

Asymptomatic malaria infection

Regardless of age group, parasite prevalence rates were similar in AA and AS individuals (Figure 1). In cohort 1 in parasitized individuals, mean parasite densities were similar within both groups of infants younger than 6 months, but increased less rapidly in AS than in AA children up to 12–17 months. In cohort 2, parasite densities were quite similar up to 36–42 months but then decreased rapidly in the AS group and plateaued in AA group (Figure 2). Mean parasite densities were significantly lower in AS than in AA children of 12–17, 48–53 and 54–59 months of age $P = 0.008$, $P = 0.004$ and $P = 0.03$, respectively.

Symptomatic malaria infection

Given the size of our cohort and the number of AS children, the number of malaria attacks observed during the follow-up was too small to allow any statistical comparison. Nevertheless, in each age group, the incidence of malaria attacks (defined as parasite density >1000 parasites/µl blood, axillary temperature >37.5°C and no symptoms relating to other infectious diseases) tended to be higher in AA subjects. The registration of hospitalized cases due to malaria (as diagnosed by the hospital physician) among children from both cohorts shows that AA children were more often admitted to the hospital than AS children (22.5% [44/195] versus 11% [5/45]), but the difference was not significant ($P = 0.22$). Moreover, according to information collected from medical reports, AA children more frequently had high (>10 000 parasites/µl) parasite densities (16 AA subjects, 1 AS subject), severe malaria (defined as asexual parasitaemia with one or more following symptoms: coma or prostration, respiratory distress, sever anaemia) (13 AA subjects) and cerebral malaria (defined as asexual parasitaemia with coma, repeated generalized convulsions) (2 AA subjects) than AS children.

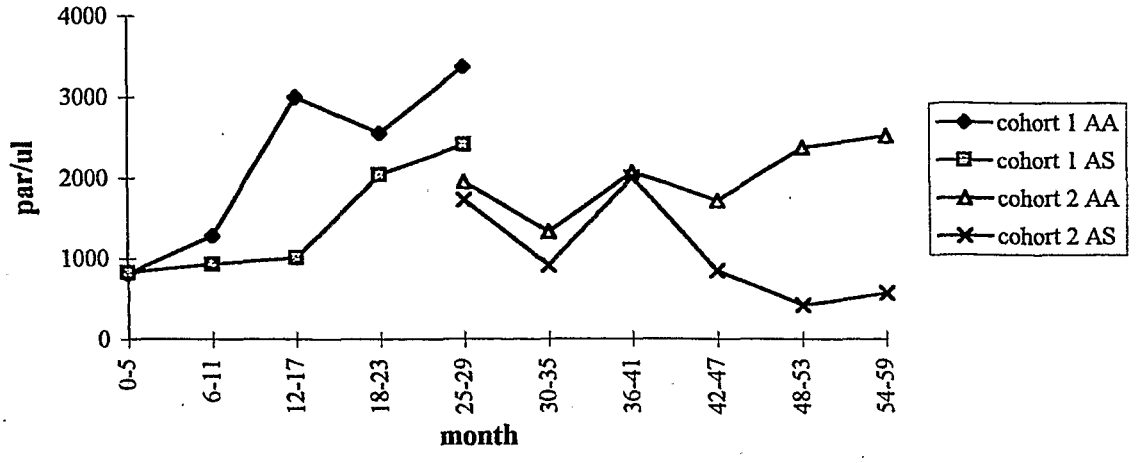

Figure 2 Six monthly evolution of geometric mean parasite densities in children of the two cohorts presenting with AA: cohort 1 (—◆—), cohort 2 (—△—) or AS: cohort 1 (—■—), cohort 2 (—✕—) haemoglobin

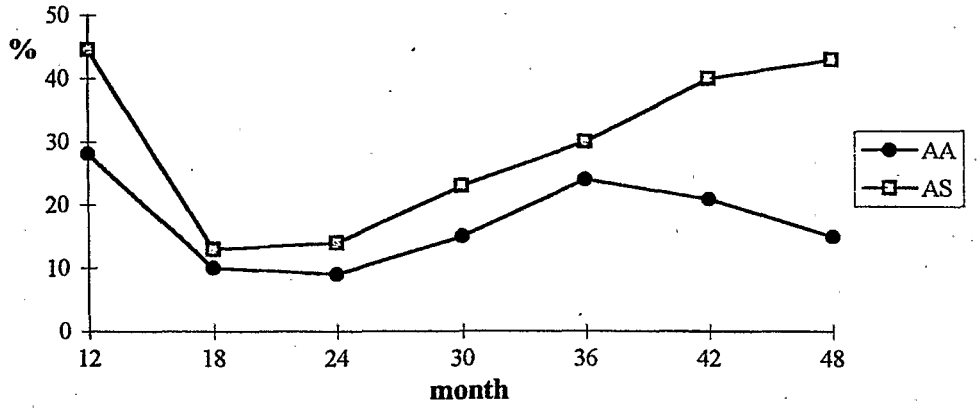

Figure 3 Six monthly evolution of prevalence of lymphocyte proliferative responses to the Pf155/RESA antigen of *Plasmodium falciparum* in children with AA (—●—) or AS (—□—) haemoglobin

Immune response

Anti-*P. falciparum* antibody rates gradually increased from 44.7% (at 6 months) to a plateau at 90–95%. This plateau was reached at 18 months of age in AA children, and at 30 months in AS children. The prevalence rates of anti-*P. falciparum* antibody were higher in AA than in AS children at the age of 12 (61.2% versus 37.5%; $P = 0.04$) and 18 (90.0% versus 65.0%; $P = 0.005$) months. Similarly, anti-Pf155/RESA antibody, as measured by EMIF, increased from 50.6% (at 6 months) to a plateau at 80–90%. Again, this plateau was reached earlier in AA (at 36 months) than in AS (at 48 months) children; the prevalence rate being significantly higher in AA children from 12 to 42 months of age.

The rates of cellular responders to non-specific antigens such as leucoagglutinin and PPD were similar in AA and AS children (leucoagglutinin: 90.5% versus 89.2%; PPD: 90.0% versus 92.8%, respectively), and did not vary with age. The overall rate of responding subjects to the Pf155/RESA antigen was higher within the AS group (26.0%) than within the AA group (16.5%). The low number of responding subjects did not allow efficient testing of any discrepancy between AA and AS within each age group. However, the variation in Pf155/RESA responder rates from 12 months to 4 years old (Figure 3) shows an evolution of immunity taking place in several steps and with some time-lag between the AA and AS children. Initially there was a decrease in the prevalence rate of responding subjects between 12 and 18 months but past the age of 24 months, the number of responding subjects increased again. It increased up to 48 months old for the AS group but decreased slowly for the AA group after 36 months of age.

Discussion

Seven children died during the follow-up; none from malaria-related conditions. We considered as lost to follow-up 83 children in the first cohort and 40 in the second cohort. These were all children who returned to the village of their parent's origin; this is common and happens when the children reach a certain age or when they leave with their parents to go to another region of Cameroon.

The haemoglobin S gene frequency rates by ethnic group in our cohorts tally with the results published by Bodo *et al.*[14] which

showed a low frequency of the gene among populations originating from West Cameroon (Bamileke) and a high frequency rate among populations from the South (Bulu and central parts). Though the difference was not significant, the lower prevalence rate of AS individuals in the second cohort is related to a higher prevalence of Bamileke children in this cohort. Indeed, Bulu infants past a certain age (weaning age) frequently return to their villages to the care of their grandparents for financial and practical reasons.

Parasite prevalence rates were higher and parasite densities lower in cohort 2 than in cohort 1 at 25–29 months. These differences may be related to early treatment of malarial attacks and to faster care seeking in case of fever during the follow-up of children in cohort 1. Such an effect tended to appear within cohort 2 a few months after initiation of the follow-up. This illustrates the effects of our intervention and brings to light a problem that must be taken into account in cohort study analysis.

The similar malaria parasite prevalence rates in both AA and AS children, whatever their age, is in line with previous studies conducted in various parts of Africa.[5,6,7,15] This also agrees with the fact that penetration and growth of *P. falciparum* are similar in red blood cells from AA and AS subjects, at least in normal oxygenation conditions.[16]

Conversely, mean parasite density was lower in AS than in AA children after 6 months of age, as previously reported.[15,17] Indeed, haemoglobin S does not allow the correct development of *Plasmodium* in deep organs where oxygen pressure is reduced.[18] Increased clearance of parasite-infected AS erythrocytes could also account for the fact that AS children are infected as frequently as AA children, but have lower parasite density.[17] This would entail a lower rate of malaria attacks, in relation to the lower blood parasite density.[19] Our data show that there is no difference during the first 6 months of life. After this age, the difference between the two groups gradually increased with age, being highest after 4 years of age. Before the age of 6 months, parasite densities were similar in both groups, probably because the action of haemoglobin S was covered by the protective effects of both maternally-transmitted antibodies[20] and the persistence of haemoglobin F, which curbs the growth of the parasite.[21] The age-related development of anti-malarial immunity is adding up to the potential protective effect of haemoglobin S, but also to cover it due to the relative importance of both mechanisms. According to Allen *et al.*,[11] the effect of haemoglobin S would rapidly be concealed by malaria premunition after 6 years of age, whose efficiency is similar whatever the haemoglobin status. Several studies demonstrated that, before 6 years of age, the immune system of AS and AA children did not respond in the same way to parasite infections.[11] *P. falciparum* has the capacity to get round the host's defences through antigenic polymorphism and/or misleading and disturbing the host's immune system. These escape mechanisms would be triggered off only in case of a high parasite density, which occurs less frequently in AS subjects than in AA children. Beside these parasite defence mechanisms, immunological processes peculiar to AS children may be involved, such as the triggering of lymphocytes by falciformed red blood cells that may lead to a modification of parasite antigens, thus revealing additional epitopes.[22] Additionally, studies of *P. falciparum* polymorphism have suggested that multiple infections were more frequent in AS than in AA individuals (*Ntoumi, personal communication*); a phenomenon likely to explain the faster acquisition of protective immunity in sickle cell trait presenting individuals.

The few cases hospitalized due to malaria most frequently involved AA subjects. Such findings corroborate those of Colombo and Felicetti[23] who reported that the hospitalization rate in a malaria-endemic area was 35% less among sickle cell trait sufferers than among AA individuals. Similarly, both the frequency and the severity of malaria attacks were noticeably higher among AA than among AS subjects.[6] Overall, it has been postulated that haemoglobin S grants 90% protection against severe or cerebral malaria, and 60% protection against non-severe malaria.[24,25]

Populations were generally well acquainted with anti-malaria prophylaxis. Most parents (57%) claimed they gave some preventive drugs, almost always (95%) chloroquine. This might be a confounding factor in our analysis but only 16% (39/244) of families specified giving the drug regularly, at the right dosage, and were able to prove this. To assess the efficacy of drug prophylaxis, we compared the proportion of blood smears positive for malaria parasites among children of these 39 families and those from the other families. Our results showed that drug prophylaxis does not appear to result in a notable reduction in parasite prevalence rates (personal data). However, the effect of drug prophylaxis remains difficult to account for in the analysis, given the poor reliability of the questioning and the lack of constant behaviour throughout the duration of study. Moreover, the high level of drug resistance of *P. falciparum* to chloroquine in the population would tend to abrogate any chemoprophylatic effect. Lastly, the proportion of children using drug prophylaxis was the same in AA and AS groups. Bednets are not widely used in the study area. Nevertheless, 25% of mothers said they used cot bednets, albeit irregularly. This figure declined rapidly with age falling to zero at the age of 2 years.

In order to investigate the basis of the protection due to sickle cell trait, we compared the immune response of AA and AS children. Anti-*P. falciparum* humoral and cellular immune responses were apparently discrepant, as the prevalence of antibody response was higher (or increased more rapidly), and that of cellular response was lower (or increased less rapidly) in AA than in AS children. No such difference was observed with the cellular responses to non-specific stimulants (leucoagglutinin and PPD), that did not vary with age or haemoglobin phenotype, as previously reported.[11,26] This apparent discrepancy may be related to the fact that, after one year of age and over a period of a few years, both blood parasite densities and incidence of malaria attacks are considered to be at their lifetime highest.[19] During this period, the immune system is highly stimulated, and may be progressively overwhelmed by an immunosuppression process, as occurs following periods of intense malaria transmission. Indeed, seasonal variations in the immune response, with humoral response enhancement and cellular response suppression at the end of the rainy season, have been reported.[26,27] Given the lower parasite densities observed in AS children during their first years of life, these variations are likely to be less intense among AS children.[10,26] Hence, infants with the sickle cell trait appear to enjoy a reduced humoral response and a reinforced cell-mediated response, as compared to AA children. Whether these variations in the immune response to *P. falciparum*, and in particular to Pf155/RESA, are responsible

for the protection of AS children against the malaria disease remains to be established. Whether this difference in immune response of AS children vanishes once premunition is acquired should also be assessed. Indeed, all attempts to confirm the protection granted by the S gene against *P. falciparum* malaria among schoolchildren and adults have failed, probably due to the effect of immunity acquired by people living within endemic areas.

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