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Understanding the Mechanisms by Which Sickle Cell Trait Protects Against Malaria

By

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A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science in Health and Medical Sciences in the Graduate Division of the University of California, Berkeley

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Part I: Understanding the Mechanisms by Which Sickle Cell Trait Protects Against Malaria

Introduction

Malaria, especially that caused by *Plasmodium falciparum*, has been a major cause of morbidity and mortality throughout human history. As a result, malaria has exerted extraordinary evolutionary pressure on the human genome and appears to have selected for multiple genetic polymorphisms that provide protection against severe disease (Kwiatkowski & Luoni, 2006; Roberts DJ, Harris, T & Williams, T, 2004; Williams, 2006). The best-characterized human genetic polymorphism associated with malaria is sickle hemoglobin (HbS). The high prevalence of HbS in sub-Saharan Africa and some other tropical areas is almost certainly due to protection of heterozygotes against malaria (Kwiatkowski & Luoni, 2006; Roberts DJ et al., 2004; Williams, 2006). Since the protective effect of sickle cell trait on malaria was first described over 60 years ago (Allison, A.C., 1954; Beet, EA, 1946; Brain, 1952), our understanding of the epidemiology and mechanisms of protection of this genotype have continued to expand, as will be discussed below.

Sickle Hemoglobin, Sickle Cell Disease, and Sickle Cell Trait

Sickle hemoglobin (HbS) is a structural variant of normal adult hemoglobin. Adult hemoglobin (HbAA) is made up of two alpha and two beta globin chains. HbS is the result of a single point mutation (Glu→Val) on the sixth codon of the beta globin gene. Homozygotes for hemoglobin S (HbSS) with two affected beta chains develop
sickle cell disease, in which polymerized hemoglobin causes red blood cells to sickle and occlude blood vessels. Vasoocclusion affects many organs and tissues, and results in high morbidity and mortality. Heterozygotes for sickle hemoglobin (HbAS) have sickle cell trait and are generally asymptomatic (Steinberg, MH & Nagel, RL, 2001).

**HbAS and Protection against Malaria**

Despite the obvious deleterious nature of HbSS, it is now widely accepted that the persistence of the sickle mutation in human populations is due to the protection from malaria afforded to heterozygous individuals. JBS Haldane first proposed the concept of a heterozygote advantage against malaria in 1949 (Haldane, JBS, 1949). In his seminal paper, Haldane suggested that individuals heterozygous for thalassemia, another hemoglobinopathy, were protected against malaria (Haldane, JBS, 1949).

Contemporaneous to this hypothesis, epidemiologic evidence for protection against malaria in those with HbS was emerging. In 1946, a British medical officer reported that the prevalence of malarial parasitemia was lower in a cohort of “sicklers”, compared to “non-sicklers” in Northern Rhodesia (Beet, EA, 1946), findings corroborated a few years later in another study in 1952 (BRAIN, 1952). The most convincing and thorough findings linking HbAS and protection against malaria came from Uganda in 1954. This study showed a reduced prevalence of parasitemia in Ugandans with HbAS compared to those with HbAA (Alison 1954). In addition, after
experimental infection Ugandans with HbAS had reduced parasitemia and less clinical malaria than those with HbAA. Since these observations, strong evidence for the protective effects of HbAS against malaria has been generated in multiple case control and cohort studies (Gilles et al., 1967; Hill et al., 1991; Jallow et al., 2009; May et al., 2007; Williams et al., 2005b). Furthermore, evidence for the protective effects of other red blood cell polymorphisms against malaria, including hemoglobin C, hemoglobin E, thalassemias, and ovalocytosis have also been described (Kwiatkowski & Luoni, 2006; Roberts DJ et al., 2004; Williams, 2006).

In endemic countries infection with *P. falciparum* causes a range of outcomes, from asymptomatic parasitemia to uncomplicated disease to severe malaria, which commonly progresses to death. HbAS provides significant protection against both severe and uncomplicated malaria. Case-control and cohort studies in multiple African countries have consistently found that HbAS is 70 to 90 percent protective against severe malaria (Gilles et al., 1967; Hill et al., 1991; Jallow et al., 2009; May et al., 2007; Williams et al., 2005b) and 75 percent protective against hospitalization for malaria (Williams et al., 2005b). In addition, a cohort study showed a 60 percent reduction in overall mortality in children aged 2 to 16 months in an area of high malaria transmission (Aidoo et al., 2002). Children with HbAS were also protected from uncomplicated malaria, with cohort studies showing that HbAS is about 50 percent protective against uncomplicated malaria in children (Clark et al., 2008; Crompton et al., 2008; Kreuels et al., 2010; Marsh, Otoo, Hayes, Carson & Greenwood, 1989; Williams et al., 2005b). Cohort and cross sectional studies also
found lower parasite densities during symptomatic malaria in HbAS subjects compared to HbAA subjects (Aidoo et al., 2002; Crompton et al., 2008; Guggenmoos-Holzmann, Bienzle & Luzzatto, 1981; Marsh et al., 1989; Mockenhaupt et al., 2004; Williams et al., 2005b), which suggests that the protective effects of HbAS may be due to better control of parasitemia. Data on the multiplicity of infection, the number of genetically distinct parasites causing a simultaneous infection, are limited, and results have been conflicting (Konaté et al., 1999; Mockenhaupt et al., 2004; Ntoumi et al., 1997). Available cross sectional studies have not found HbAS to be protective against asymptomatic parasitemia (Fleming, Storey, Molineaux, Iroko & Attai, 1979; Jakobsen et al., 1991; Marsh et al., 1989; Roberts DJ et al., 2004; Williams et al., 2005b). Putting these results together, HbAS protects against severe and uncomplicated malaria, possibly through better control of parasitemia, but does not appear to protect against asymptomatic infection.

**Molecular Mechanism of Protection**

Some decades ago, investigators found that *P. falciparum* parasites induced sickling of HbAS red blood cells in vitro. In the 1970's, two groups showed that parasitized HbAS cells sickled at a two to eight times higher rate than non-parasitized cells (Luzzatto, Nwachuku-Jarrett & Reddy, 1970; Roth et al., 1978). One group also visualized polymerized hemoglobin in parasitized red blood cells and hypothesized that an increase in the polymerized hemoglobin or a reduced intracellular pH might cause increased sickling (Roth et al., 1978). Increased sickling of parasitized red blood cells in HbAS individuals may promote increased phagocytosis of the infected
cells, and therefore result in reduced parasitemia compared to that in HbAA individuals.

Later in the 1970's, multiple studies found that *P. falciparum* ring-stage parasites did not grow in HbAS red blood cells at low oxygen tension (Friedman, 1978; Pasvol, Weatherall & Wilson, 1978; Roth et al., 1978). Parasite growth was inhibited in both sickled and non-sickled HbAS red blood cells (Pasvol et al., 1978), suggesting that factors in addition to sickling affected parasite growth. It has been hypothesized that specific intraerythrocytic conditions of HbAS red blood cells such as low intracellular potassium (Friedman, 1978), high concentrations of hemoglobin (Orji, Chevli & Fitch, 1985), or osmotic shrinkage of the red blood cell (Ginsburg, Handeli, Friedman, Gorodetsky & Krugliak, 1986) cause an inhospitable environment for parasites. A study also demonstrated that *P. falciparum* parasites invaded HbAS red blood cells less efficiently than HbAA cells at low oxygen tension (Luzzatto et al., 1970), but subsequent studies failed to replicate these results (Ayi, Turrini, Piga & Arese, 2004; Roberts, DJ, Harris, T & Williams, T, 2004).

Biochemical and mechanical changes in infected HbAS red blood cells have been shown to alter disease progression. Rosette formation, which is the binding of *P. falciparum* infected red blood cells to uninfected red blood cells, is thought to lead to microcirculatory obstruction in cerebral malaria (Aikawa et al., 1990; Carlson et al., 1990; Trager, Rudzinska & Bradbury, 1966; Treutiger et al., 1992; Udomsangpetch et al., 1989). Rosette formation was impaired in *P. falciparum* infected HbAS red
blood cells under deoxygenated conditions (Carlson, Nash, Gabutti, al-Yaman & Wahlgren, 1994). Impaired rosette formation with HbAS red blood cells may be due to increased sickling of these cells in deoxygenated conditions (Luzzatto et al., 1970; Roth et al., 1970) or to reduced expression of erythrocyte surface adherence proteins (Cholera et al., 2008).

Reduced cytoadherence has been implicated as a mechanism of protection in HbAS individuals. Infected red blood cells express one of a family of parasite-encoded PfEMP-1 molecules on the erythrocyte surface, and via this protein adhere to endothelial cells in the microvasculature (McGilvray, Serghides, Kapus, Rotstein & Kain, 2000; Ockenhouse & Shear, 1983; Ockenhouse, Tandon, Magowan, Jamieson & Chulay, 1989; Yazdani, Mukherjee, Chauhan & Chitnis, 2006). This process, termed cytoadherence, enables parasites to sequester in the vasculature and avoid clearance by the spleen (Yazdani et al., 2006). Cytoadherence also leads to endothelial activation and associated inflammation in the brain and other organs, and thereby the progression of severe malaria (Dondorp, Pongponratn & White, 2004; Kaul, Roth, Nagel, Howard & Handunnetti, 1991; Miller, Baruch, Marsh & Doumbo, 2002; Turner et al., 1994). Reduced cytoadherence was first seen after infection of red blood cells with another hemoglobinopathy, HbC (Fairhurst et al., 2005), which is due to a different mutation (Glu→Lys) at the same site on the beta chain affected in HbS. Altered expression of PfEMP-1 was subsequently found in HbAS red blood cells in vitro (Cholera et al., 2008). Comparison of binding properties showed reduced adherence to endothelial cells expressing the binding
ligand CD36 compared to HbAA red blood cells. PfEMP-1 surface signal was reduced by 14 percent in HbAS and HbSS compared to HbAA erythrocytes in flow cytometric assays, suggesting altered surface expression of PfEMP-1 (Cholera et al., 2008). Oxidative stress in HbAS red blood cells has been hypothesized to lead to increased membrane-bound hemichromes, aggregated band 3, and autologous IgG and complement C3c fragments (Ayi et al., 2004). These changes may impair parasite induced remodeling of the red blood cell surface membrane and lead to altered PfEMP-1 surface expression (Cholera et al., 2008; Fairhurst et al., 2005). Reduced cytoadherence of HbAS and HbSS erythrocytes leading to increased splenic clearance may in part explain lower parasite densities in individuals with HbAS and, most importantly, the lower incidence of severe malaria in HbAS individuals.

**Role of the Innate Immune System**

Phagocytosis by monocytes of HbAS red blood cells infected with *P. falciparum* was found to be enhanced compared to that of infected HbAA cells, potentially explaining the protection against *P. falciparum* in HbAS individuals (Ayi et al., 2004). Enhanced phagocytosis may be due to increased presentation of opsonins, including membrane bound IgG, C3c, membrane-bound hemichromes, and aggregated band 3 (Destro-Bisol G, Giardina, B, Sansonetti, B & Spedini, B, n.d.). These opsonins, which are thought to be involved in the removal of senescent red blood cells, were first shown to be increased in G6PD deficiency (Arese, Turrini & Schwarzer, 2005; Cappadoro et al., 1998), a red blood cell enzyme deficiency also
protective against malaria (Roberts DJ et al., 2004; Williams, 2006), and were also significantly higher in infected HbAS compared to HbAA red blood cells. Clearance by monocytes of red blood cells with exposed phosphatidylserine, a surface marker of damaged erythrocytes (Boas, Forman & Beutler, 1998; Hoffmann et al., 2001; Lang et al., 2009; Schroit, Madsen & Tanaka, 1985; Tanaka & Schroit, 1983), was also enhanced in infected HbAS compared to HbAA cells (Lang et al., 2009).

Enhanced opsonization and clearance of parasitized HbAS red blood cells in the spleen could lead to increased antigen presentation and earlier development of acquired immunity compared to that in HbAA individuals. A cross sectional study found decreased levels of peripheral myeloid dendritic cells and monocytes in individuals with HbAS during healthy periods and malaria (Urban et al., 2006), suggesting increased monocyte and dendritic cell recruitment to the spleen.

**Role of the Acquired Immune System**

Population studies have found that the protective effect of HbAS increases with age, suggesting an acquired immune component of protection. A cross sectional study of children with malaria in Nigeria found a significantly lower mean parasite density in HbAS compared with HbAA children in those 2 to 4 years old, but not in children less than 2 years old (Guggenmoos-Holzmann et al., 1981). In Kenyan children, protection afforded by HbAS against symptomatic malaria increased from 20 percent in children less than 2 years old to a peak of 56 percent by age 10 years (Williams et al., 2005a). Preliminary data found by our group also suggest enhanced
protection from malaria in older children. In a cohort of children aged 1 to 10 years old in Kampala, Uganda (Clark et al., 2010), we found that protection from symptomatic malaria once parasitemic significantly increased with age (unpublished). Several studies shed light on possible immune bases for these findings.

**Role of Cell Mediated Immunity**

Cell mediated responses to *P. falciparum* appear to be increased in HbAS compared to HbAA individuals. The mean lymphoproliferative response to affinity-purified *P. falciparum* soluble antigens (Bygbjerg, Jepsen, Theander & Odum, 1985; Jepsen & Andersen, 1981; Riley, Andersson, Otoo, Jepsen & Greenwood, 1988) was found to be significantly higher in HbAS children compared to HbAA children (Abu-Zeid et al., 1991; Abu-Zeid et al., 1992; Bayoumi et al., 1990; Le Hesran et al., 1999), but a significant difference has not consistently been found between HbAA and HbAS adults (Abu-Zeid et al., 1991; Bayoumi et al., 1990). Although available results suggest a more robust cellular response to *P. falciparum* in HbAS children, it is unclear whether this is a cause or effect of the protective effects of HbAS. The lymphoproliferative response is suppressed during and after acute malarial infection in HbAA individuals (Angulo & Fresno, 2002; Brasseur et al., 1983; Ho et al., 1986; Riley et al., 1988; Theander et al., 1986; Yazdani et al., 2006). Therefore, a more robust lymphoproliferative response in HbAS individuals could be secondary to decreased malaria and thus less malaria-induced suppression.
Role of Humoral Immunity

Investigators have also found evidence for an enhanced humoral response in subjects with HbAS. Increased levels of gamma globulin were found in HbAS compared to HbAA children (Cornille-Broger, R, Flemming, AF & Kagan, 1979; Edozien, Boyo & Morley, 1960). However, higher levels of specific antibodies directed at antigens located on the surface of the parasite and believed to play a role in protective humoral responses, including Pf155, RESA, MSP1, MSP2, and GLURP, have not been seen in HbAS compared to HbAA individuals (Marsh 1989; Dziegel 1993; Aluoch 1997; Le Hesran 1999; Aucan 2000; Luty 2000). Indeed, one preliminary study found decreased antibodies toward free parasite antigens AMA1, MSP1, MSP2, and EBA175 associated with HbAS (Kazutoyo, M. 2010 ASMTH abstract), most likely due to less infection with malaria and resultant reduced exposure to free parasite antigens in HbAS individuals.

In contrast, higher levels of IgG directed at PfEMP-1 family proteins, which are located on the surface of the red blood cell, have been found in individuals with HbAS in some studies. In the Gambia (Marsh et al., 1989), Gabon (Cabrera et al., 2005), and a low transmission area of Burkina Faso (Verra et al., 2007), HbAS children had higher levels of IgG antibodies toward PfEMP-1 than did those with HbAA (Marsh et al., 1989). However, other studies failed to find increased antibody response to PfEMP-1 in HbAS children in high malaria transmission areas (Allen et al., 1992; Verra et al., 2007), perhaps because in these high malaria transmission areas robust responses were seen in all children. The identification of high levels of
IgG toward PfEMP-1 and not toward other parasite antigens suggests that an enhanced humoral immune response is directed specifically at proteins on the surface of the infected red blood cell. This phenomenon may be due to increased splenic uptake of infected red blood cells in HbAS individuals and therefore improved presentation of surface antigens. High levels of antibodies to PfEMP-1 may mediate protection in HbAS individuals via enhanced opsonization and phagocytosis of infected red blood cells, or through destabilization of cytoadherence. Accelerated acquisition of antibodies to PfEMP-1 may therefore underly the age-dependent increase in protective effects of HbAS found in two studies (Guggenmoos-Holzmann et al., 1981; Williams et al., 2005b).

**Perspectives**

It is likely that both biochemical and immune mechanisms contribute to the protection afforded against falciparum malaria by the HbAS genotype. HbS clearly induces biochemical changes in the red blood cell that may affect parasite metabolism and growth. *P. falciparum* also likely inflicts oxidative damage on the HbAS red blood cell membrane resulting in altered PfEMP-1 expression and reduced cytoadherence. Reduced cytoadherence could lead to decreased endothelial activation and decreased inflammation implicated in the pathogenesis of severe malaria. In addition, it could also lead to increased splenic uptake of infected erythrocytes. Within the spleen, increased antigen presentation may lead to accelerated development of antibodies to PfEMP-1. Higher levels of antibodies directed against PfEMP-1 and possibly other surface proteins might enhance
opsonization and phagocytosis of infected red blood cells and further destabilize the
cytoadherence properties of infected red blood cells. Together, these mechanisms
may lead to better control of parasitemia in HbAS children and protect against both
uncomplicated and severe malaria. Knowledge of these mechanisms gives insight
into the host-parasite relationship and more specifically how *P. falciparum* interacts
with the human immune system.
Part II: Sickle Cell Trait Alters *Plasmodium falciparum* Infection Dynamics and Accelerates Acquisition of Protection Against Malaria

**Introduction**

Malaria caused by *Plasmodium falciparum* continues to be a major cause of child morbidity and mortality in sub-Saharan Africa (World Health Organization, n.d.). Throughout history, malaria has selected for host genetic polymorphisms that provide protection against severe disease. Indeed, the high prevalence of the sickle hemoglobin gene in sub-Saharan Africa is generally thought to be due to the survival advantage conferred by its carrier form sickle cell trait (HbAS) that offers protection against severe (Aidoo et al., 2002; Gilles et al., 1967; Hill et al., 1991; Jallow et al., 2009; May et al., 2007) and uncomplicated malaria (Clark et al., 2008; Crompton et al., 2008; Williams et al., 2005b).

Although the protective effect of HbAS has been well known for over 60 years, the mechanism of protection remains unclear. Epidemiologic studies have found similar rates of asymptomatic parasitemia in HbAS and non-sickle cell trait (HbAA) children (Roberts DJ et al., 2004; Williams et al., 2005b) suggesting HbAS does not provide protection against the establishment of patent parasitemia. However, a number of studies have found depressed parasite densities during malaria in HbAS individuals suggesting HbAS may enhance control of parasitemia once patent infections are established (Abu-Zeid et al., 1992; Aidoo et al., 2002; Crompton et al., 2008; Marsh et al., 1989; May et al., 2007; Mockenhaupt et al., 2004; Williams et al., 2005b).
In vitro studies have found reduced intraerythrocytic parasitic growth (Friedman, 1978; Pasvol et al., 1978) and altered surface proteins on infected erythrocytes resulting in reduced cytoadherence (Cholera et al., 2008) and potentially leading to enhanced clearance in HbAS individuals (Ayi et al., 2004) that may at least in part explain enhanced control of parasitemia. Investigators have also found a trend towards increased protection by HbAS with age suggesting an immune component of protection (Guggenmoos-Holzmann et al., 1981; Williams et al., 2005b). Cytoadherence properties may be further destabilized by a more robust humoral immune response in HbAS individuals found in several studies (Cabrera et al., 2005; Marsh et al., 1989; Verra et al., 2007). However, it remains unclear how HbAS alters *P. falciparum* infections *in vivo* at the level of an individual strain. Few studies have investigated *P. falciparum* infection dynamics in HbAS children. Data on the multiplicity of infection, the number of strains detected during a parasitemic episode, have been conflicting (Konaté et al., 1999; May et al., 2007; Mockenhaupt et al., 2004; Ntoumi et al., 1997; Vafa, Troye-Blomberg, Anchang, Garcia & Migot-Nabias, 2008). Furthermore, to our knowledge, no other longitudinal studies have followed patent blood stream infections and therefore little is known about the acquisition, duration, and clearance of infections in HbAS children.

To address how HbAS alters *P. falciparum* infection dynamics, a cohort of children in Kampala, Uganda was followed for 18 months using molecular genotyping techniques to identify and follow *P. falciparum* infections. The effect of HbAS on the probability of parasitemia and malaria, force of infection (acquisition of parasite
strains), multiplicity of infection (number of strains present during a parasitemic episode), parasite density, duration of infection, and spontaneous clearance (clearance of parasites in the absence of treatment) was evaluated. Lastly, the effect of age was analyzed to investigate an immune component of protection.

**Materials and Methods**

*Study site and participants.* The study was conducted in a cohort of 601 children living in Mulago III Parish of Kampala, Uganda, a densely populated urban slum where Malaria is mesoendemic. The details of the study cohort have been published elsewhere (Clark et al., 2008; Clark et al., 2010; Dorsey et al., 2007). Between November 2004 and April 2005, children aged 1 to 10-years-old were randomly selected from a census performed in a geographically defined area (Davis et al., 2006) and enrolled in a randomized trial of combination antimalarial therapies. The cohort was followed until June 2006. Hemoglobin type was determined at enrollment by hemoglobin electrophoresis and children with sickle cell disease (HbSS) were excluded from the study. Parents or guardians of study participants were asked to bring their children to a designated study clinic for all medical care. Each time a child presented to the clinic with a fever, defined as the subjective history of a fever or a temperature greater than 38 degrees Celsius, a thick blood smear was performed. Malaria was diagnosed if a child had a fever and a thick blood smear positive for parasites and was subsequently treated with combination therapy. All episodes of malaria were treated with directly observed combination therapy and actively followed for 28 days to ensure response to therapy (Dorsey et
Children also had a blood smear performed every 30 days for detection of asymptomatic parasitemia.

*Genotyping by capillary electrophoresis.* To identify *P. falciparum* strains present at the time of infection, all filter paper samples at the time of all positive blood smears for malaria or asymptomatic parasitemia were genotyped. To determine parasite genotypes, the highly diverse polymorphic surface antigen *merozoite surface protein* 2 (*msp2*) was sized by capillary electrophoresis. The genotyping protocol used in this study has been published and we have shown that the probability of 2 genotypes matching by chance is less than 3% (Gupta, Dorsey, Hubbard, Rosenthal & Greenhouse, 2010). Briefly, DNA was extracted from stored blood spots on filter paper into 130 μl of water using a standard Chelex protocol (Plowe, Djimde, Bouare, Doumbo & Wellems, 1995). DNA was then amplified using nested PCR with second round primers specific for the *msp2* allelic families (FC27 and IC3D7). Amplified PCR products were denatured and sized on an Applied Biosystems 3730xl DNA analyzer. Alleles were called with GeneMapper 4.0 software (Applied Biosystems). Two alleles were considered the same if the size of *msp2* was within 1 base. Laboratory artifact was distinguished from true alleles by a single caller who was blinded to the status of the samples. Questionable alleles were labeled as such by the caller and were automatically removed if the same size allele was not called as a true allele in contiguous samples from the same subject. A strain was considered cleared if it was present in a sample and not detected in two subsequent, contiguous samples. Strains were considered persistent if they were detected in
consecutive, contiguous samples allowing for one "skip" if a sample was detected in two of three contiguous samples and the intervening sample was either negative for parasites or failed genotyping. Failed reactions were repeated once, after which they were classified as having failed genotyping.

Statistical analysis. All analyses were performed considering our main exposure hemoglobin type as a binary variable, having sickle cell trait (HbAS) or not having sickle cell trait (HbAA). Interaction with age, considered as a continuous variable, was also evaluated in all analyses. All analyses were performed using generalized estimating equations to account for clustering within individuals except force of infection that was determined by negative binomial regression.

To assess the association between hemoglobin type and blood-stage immunity, we evaluated the risk of developing malaria once parasitemic in a given month. Asymptomatic parasitemia was defined as the presence of a positive blood smear in the absence of fever at least 5 days before and 28 days after a patient received treatment for malaria. Outcomes were each month of person-time classified as containing at least one episode of malaria (no protection), no malaria but at least one episode of asymptomatic parasitemia (protection), or neither (no evidence for or against protection). Months were defined by calendar time and all months in which at least one blood smear was taken were included in our analysis. Outcomes were assessed monthly since each child usually had at least one blood smear evaluated every month and it was uncommon to have more than one new episode of
malaria within a month. Associations were estimated as risk ratios using
generalized estimating equations with robust inference, accounting for repeated
measures within individuals.

To assess the association between hemoglobin type and the intrahost infection
dynamics, force of infection, multiplicity of infection, duration of infection, and
spontaneous clearance of infections were evaluated. Force of infection was defined
as incident parasite strains per person-year at risk assessed as the clonal acquisition
rate (Falk 2006). Time at risk excluded the 14 days following treatment for a
malaria episode. Multiplicity of infection was defined as the number of unique
strains detected by genotyping of a positive blood smear, evaluated during episodes
of malaria and asymptomatic parasitemia. Duration of infection was defined as the
number of days between the first positive blood smear and the last positive blood
smear during which a parasite strain was detected. In addition, we assumed an
asymptomatic infection was present both 15 days before and 15 days after a blood
smear was taken and an infection that presented as malaria was present 4 days
before a child presented with symptoms. Spontaneous clearance was defined as the
inability to detect a parasite strain in 2 contiguous blood smears in the absence of
treatment. Our outcomes of force of infection, multiplicity of infection, and duration
of infection were evaluated as a continuous variables and our outcome of
spontaneous clearance was evaluated as a binary variable. For force of infection, the
association was estimated as an incidence rate ratio using negative binomial
regression. For multiplicity of infection and duration of infection, associations were
estimated as relative changes using generalized estimating equations to account for repeated measures within individuals. For spontaneous clearance, the association was estimated as a risk ratio using generalized estimating equations.

To assess the association between hemoglobin type and control of parastemia the relative change in parasite density during malaria and asymptomatic parasitemia was evaluated. Our outcome was the natural log of parasite density evaluated as continuous variables during malaria, and asymptomatic parasitemia. Only blood smears positive for parasites were included in the analysis. Associations were estimated as relative changes using generalized estimating equations with robust inference, accounting for repeated measures within individuals.

Statistical analysis was performed using Stata (version 9; Stata) and R version 2.9.0 (R Foundation for Statistical Computing, Vienna, Austria). A P value of 0.2 was considered significant for interaction terms; a P value of 0.05 was considered significant for all other analyses.

*Ethical Permission.* All study participants or their parents or guardians provided individual written informed consent. Ethical permission was granted by the Uganda National council of Science and Technology, the Makerere University Research and Ethics Committee, the University of California Committee on Human Research, and the University of California Berkeley Committee for Protection of Human Subjects.
RESULTS

Cohort Characteristics. Of 601 children enrolled in the study, 99 (16.5%) had HbAS (Figure 1). Variables previously associated with risk of malaria in this cohort such as age, use of insecticide treated bed net, and distance of residence from a mosquito breeding site (Clark et al., 2008) were similar between HbAA and HbAS children (Table 1). Between November 2004 and June 2006, children were followed for a total of 9,644 months of person-time, with a median of 16.8 months per child. A total of 377 children (62.7%) were found to have at least one blood sample positive for malaria parasites and 350 (58.2%) were diagnosed with at least one episode of malaria (Figure 1).

Relationship between HbAS and probability of parasitemia and malaria. We have previously shown that the incidence of malaria was lower in HbAS children in this cohort (RR 0.70, 95% CI [0.52-0.95], P=0.007) (Clark et al., 2008). To determine whether children with HbAS were protected against parasitemia, malaria, or both, the probability of being parasitemic and having malaria for each month of follow-up was assessed. HbAA and HbAS children had the same probability of being parasitemic in a given month but HbAS children were 19% less likely to develop malaria once parasitemic (Table 2). In addition, the monthly risk of developing malaria if parasitemic decreased with increasing age in both HbAA children (RR 0.96 per 1 year increase in age, 95%CI [0.95-0.98], P<0.001) and HbAS children (RR 0.92 per 1 year increase in age, 95%CI [0.87-0.97], P=0.002). However, protection
increased more rapidly with age in HbAS children (P value for interaction = 0.15), (Figure 2). Statistical modeling estimated that HbAS children were 8% less likely than HbAA children to develop malaria once parasitemic at 2 years of age (RR 0.92, 95%CI [0.77-1.10], P=0.34) but this difference increased to 32% by 9 years of age (RR 0.68, 95%CI [0.51-0.91], P=0.008).

*Relationship between sickle cell trait and force of infection and multiplicity of infection.* To better understand the underlying mechanism behind our clinical findings, the intrahost infection dynamics of *P. falciparum* from the time patent parasitemia was first detected to the clearance of infections either spontaneously or following antimalarial therapy were characterized. To assess differences in the acquisition of blood-stage *P. falciparum* infections in HbAS and HbAA children, *P. falciparum* strains in children with parasitemia were identified using high-resolution genotyping of every parasitemic episode. Genotyping was successful for 1,325 of 1,438 filter paper samples (92.1%) meeting study criteria. The force of infection, defined as the number of new strains detected in an individual per year, was evaluated in HbAA and HbAS children. There was a trend towards a reduced force of infection in HbAS children (IRR 0.75, 95%CI [0.51-1.08], P=0.124). Interestingly, HbAS also altered the relationship between age and force of infection (p value for interaction = 0.11). Force of infection was not modified by age in HbAA children (IRR 1.03, 95%CI [0.97-1.10], P=0.317), whereas there was a trend towards a decreasing force of infection with increasing age in HbAS children (0.91, 95%CI [0.80-1.03], P=0.149) (Figure 3). Statistical modeling estimated that the force of
infection was similar in HbAS and HbAA children at 2 years of age (IRR 1.16, 95%CI [0.59-2.30], P=0.66) whereas the force of infection was significantly reduced in HbAS children at 9 years of age (IRR 0.50, 95%CI [0.27-0.92], P=0.025).

Consistent with the lower force of infection, the multiplicity of infection was lower in HbAS children for both episodes of malaria (Relative change in multiplicity of infection 0.80, 95%CI [0.68-0.95], P=0.011) and episodes of asymptomatic parasitemia (Relative change in multiplicity of infection 0.78, 95% CI [0.60-1.02] P=0.072) however results did not reach statistical significance during asymptomatic parasitemia (Table 3). There was no significant interaction between age and multiplicity of infection.

Relationship between sickle cell trait and parasite density. To assess enhanced control of parasitemia, parasite density was evaluated. Parasite density was lower in HbAS children during episodes of malaria (Relative parasite density 0.51, 95% CI [0.51 - 0.88], P<0.001), (Table 3). However, the difference in parasite density did not reach statistical significance during asymptomatic parasitemia (Relative parasite density 0.77, 95% CI [0.49-1.21], P=0.26). Parasite density during malaria decreased with age in both HbAS and HbAA children, but at a higher rate in HbAA children (P value for interaction 0.019), (Figure 4). Statistical modeling estimated that the relative parasite density was significantly reduced in HbAS children at 2 years of age (Relative density 0.19 95%CI [0.071-0.53], P=0.001) whereas there was
no difference in relative parasite density at 9 years of age (Relative density 1.17, 95%CI [0.52-2.66], P=0.701).

Relationship between sickle cell trait and duration of infections. To determine how long an individual parasite strain persisted in the blood, the duration of infection was evaluated. On average, parasite strains persisted approximately 19.7 days in HbAA children compared to 26.6 days in HbAS children (Difference in days 11.0 [2.69-19.34] P=0.006). There was no significant interaction between the duration of infection and age.

Relationship between sickle cell trait and spontaneous clearance of infections. Lastly, control of parasitemia by spontaneous clearance of an infection, defined as the disappearance of a parasite strain from an individual in the absence of antimalarial therapy, was evaluated. The probability of spontaneously clearing an infection was 0.31 in HbAS children compared to 0.28 in HbAA children (RR 1.45, 95%CI [1.02-2.05], P=0.036). Results were similar after adjusting for age (data not shown). There was no significant interaction between the probability of spontaneous clearance and age.

Discussion
In this study we provide detailed evidence on how HbAS alters the course of infection with *P. falciparum*. For the first time to our knowledge, we showed that HbAS children have a lower force of infection than HbAA children, demonstrating
that they acquire fewer patent blood stream infections. As a result, they have a lower multiplicity of infection, harboring fewer strains during parasitemic episodes. Once an infection is established, HbAS children are better able to control parasitemia. They have lower parasite densities and a higher probability of spontaneously clearing infections, as opposed to developing malaria, than HbAA children. Likely as a result of being treated less often for malaria, infections persist longer in HbAS children, explaining how they are parasitemic for the same number of months as HbAA children despite acquiring fewer patent blood stream infections. Lastly, we showed certain infection dynamics were modified by age, allowing HbAS children to acquire protection more rapidly than HbAA children, as they get older.

In our study, malaria transmission was assumed to be equal between HbAA and HbAS children with no evidence of increased risk of infection in either group. Under this assumption, our data suggest that HbAS children are more resistant to establishing patent blood stream infections. In HbAS children, fewer parasite strains may be able to establish patent infections and, as a result, there is a lower multiplicity of infection with fewer parasite strains present during each parasitemic episode. To our knowledge, no other studies have investigated the association between force of infection and hemoglobin type. A few studies have investigated the effect of HbAS on multiplicity of infection but results have been conflicting (Konaté et al., 1999; May et al., 2007; Mockenhaupt et al., 2004; Ntoumi et al., 1997; Vafa et al., 2008). Some studies have suggested an increased MOI with HbAS (Ntoumi et al., 1997) leading investigators to theorize that children with HbAS have
greater exposure to blood stage *P. falciparum* infections inducing greater protection. On the contrary, our results suggest that children with HbAS acquire fewer patent *P. falciparum* infections and a resultant decreased multiplicity of infection in agreement with other previous studies (May et al., 2007; Vafa et al., 2008). An alternative explanation for an increased MOI in other studies is that decreased symptomatic malaria may result in decreased treatment for malaria and accumulation of more infections despite having a lower force of infection. In addition, previous studies may have been limited by inadequate, available treatment whereas in this study, children received prompt and effective antimalarial therapy. Protection against the acquisition of blood stage *P. falciparum* infections may in part explain protection against clinical disease in HbAS children.

Once parasite strains establish patent infections, we found HbAS children are better able to control parasitemia, resulting in a lower risk of developing symptomatic malaria. Generally depressed parasite densities in HbAS children could explain reduced clinical disease since higher parasite densities are more likely to lead to symptoms. Previous studies have also consistently found lower parasite densities in HbAS children during symptomatic disease (Abu-Zeid et al., 1992; Aidoo et al., 2002; Crompton et al., 2008; Marsh et al., 1989; May et al., 2007; Mockenhaupt et al., 2004; Williams et al., 2005b). In addition, for the first time to our knowledge, we showed that once infections are established, they are more likely to be cleared in HbAS children as opposed to progressing to malaria. Increased spontaneous clearance may also be an alternative explanation for a decreased observed force of
infection as strains may be rapidly cleared before they are detected. However, we found that strains persisted longer in HbAS children making rapid clearance unlikely as the sole explanation for a lower force of infection. Our finding that patent parasite infections persisted longer in HbAS children also explains equivalent months of parasitemia in HbAA and HbAS children despite a reduced force of infection. We have previously shown that HbAS children have a lower incidence of malaria in this cohort (Clark et al., 2008). With less clinical disease, HbAS children are less likely to be treated with antimalarials, allowing strains to persist longer in the bloodstream.

Investigators have proposed multiple molecular mechanisms of protection that may at least in part explain altered strain dynamics found in our study. Early in vitro studies found decreased parasite invasion (Pasvol et al., 1978), reduced parasite growth (Friedman, 1978; Pasvol et al., 1978), and increased sickling upon parasitization of HbAS red blood cells (Luzatto 1970; Roth 1978). These changes could inhibit the ability of strains to establish patent blood stream infections in HbAS individuals or, once they do, the ability to reach high parasite densities. In addition, parasite induced changes on the surface of HbAS red blood cells may lead to reduced cytoadherence properties, inhibiting the ability of parasitized HbAS erythrocytes to sequester in post capillary microvessels and evade clearance by the spleen. Altered expression of PfEMP-1 in HbAS individuals, a surface protein with known cytoadherence properties (McGilvray et al., 2000; Ockenhouse & Shear, 1983; Ockenhouse et al., 1989; Yazdani et al., 2006) has been demonstrated in vitro
(Cholera et al., 2008). Decreased cytoadherence in HbAS children could explain increased spontaneous clearance found in our study. In addition, decreased cytoadherence may lead to increased antigen presentation in the spleen resulting in a more robust humoral immune response (Cabrera et al., 2005; Marsh et al., 1989; Verra et al., 2007).

It has been proposed that HbAS accelerates the development of acquired immunity to *P. falciparum* (Gugenmoos-Holzmann et al., 1981; Williams et al., 2005b). An older cross sectional study of children aged 9 months to 6 years found HbAS is most protective in children between 2 and 6 years old (Gugenmoos-Holzmann et al., 1981). Similarly, a more recent study found a trend towards increased protection with age from 20 percent at two years old to 56 percent at ten years old (Williams et al., 2005b). In agreement with this hypothesis, our data suggest two possible mechanisms for an immune component of protection of HbAS. In our study, we first found that HbAS provided increased protection against a high force of infection as children got older, suggesting that protection by HbAS against the establishment of a blood stage infection has an acquired immune component. We also found that once patent parasitemia is established, HbAS children develop protection against malaria more rapidly than HbAA children. However, we did not see increased control of parasite density as age increased. In fact, as children got older, parasite densities between HbAS and HbAA children became more similar. In addition, the increased ability to spontaneously clear parasites afforded by HbAS did not appear to improve in older HbAS children. Therefore, acquired protection against malaria in older
HbAS children may not be due to enhanced control of parasitemia but to prevention of patent infection and protection from symptoms once parasitemic.

In conclusion, we have provided evidence that HbAS alters *P. falciparum* infection dynamics resulting in better control of parasitemia and protection against clinical disease. We have also shown that these dynamics are modified by age resulting in enhanced protection against patent parasitemia and clinical disease once parasitemic with increasing age suggesting an acquired immune component of protection. Understanding *P. falciparum* infection dynamics could provide further insight into natural host defenses against malaria.
References


Roberts DJ, Harris, T, & Williams, T (2004). The influence of inherited traits on malaria infection. In Bellamy R (Ed.), *Susceptibility to infectious diseases: The importance of host genetics.* (pp. 139-84). Cambridge University Press.


Appendix A. Tables
Children receiving quinine for treatment of severe malaria. Four children had only 1 episode of severe malaria and were not subsequently randomized to a treatment arm.

<table>
<thead>
<tr>
<th>Quinine Arm</th>
<th>AL</th>
<th>4G + AS</th>
<th>4G + SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>82 (18.9%)</td>
<td>18 (18.2%)</td>
<td>101 (20.2%)</td>
<td>93 (18.5%)</td>
</tr>
<tr>
<td>4 (0.04%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>0.79</td>
<td>0.79</td>
<td>0.79</td>
<td>0.79</td>
</tr>
</tbody>
</table>

Never had malaria

Distance from the swamp (> 200 meters)

Insecticide treated bed net use

Bed net use

Mean age at enrollment in years (SD)

Table 1. Cohort characteristics in HbAA Children compared to HbAS Children.
| p Value (95% CI) | Relative Risk | HbA | HbS | Outcome
<table>
<thead>
<tr>
<th></th>
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<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00 [0.69-0.94]</td>
<td>0.54</td>
<td>0.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00 [0.78-1.29]</td>
<td>0.13</td>
<td>0.98</td>
<td></td>
<td></td>
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</tbody>
</table>

Table 2. HbAS does not protect against malaria but does protect against malaria once parasitic.
<table>
<thead>
<tr>
<th>Multiplicity of Infection</th>
<th>Multiplicity of Parasitemia</th>
<th>Asymptomatic Parasite Density</th>
<th><em>Malaria</em></th>
<th>P Value</th>
<th>Relative Change</th>
<th>HBAAS</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.24</td>
<td>0.76 0.47-1.19</td>
<td>70.77</td>
<td>0.49 0.29-0.85</td>
<td>0.011</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.9553</td>
<td>5661.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.072</td>
<td>0.78 0.60-1.02</td>
<td>2.08 1.11</td>
<td>2.68 [1-15]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.080</td>
<td>0.80 0.68-0.95</td>
<td>2.17 [1-8]</td>
<td>2.61 [1-15]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Multiplicity of Infection and Parasite Density in HbAAS Children Compared to HbAAS Children.
Figure 1. Distribution of study participants from enrollment to the end of the 19-month follow-up period.

Figure 2. Estimates of the probability of developing malaria in a given month if parasitemic over age-stratified by hemoglobin type.

Figure 3. Estimates of the force of infection over age-stratified by hemoglobin type.

Figure 4. Estimates of the parasite density during malaria episodes over age-stratified by hemoglobin type.
FIGURE 1.
Figure 2

Probability of Developing Malaria in a Month if Parasitemic

- HbA
- HbA