Original article

Multiclonal asymptomatic Plasmodium falciparum infections predict a reduced risk of malaria disease in a Tanzanian population

Sándor Bereczky a, Anne Liljander a, Ingegerd Rooth d, Lea Faraja d, Fredrik Granath b, Scott M. Montgomery b,c, Anna Färnert a,*

a Infectious Diseases Unit, Department of Medicine, Karolinska Institute, Karolinska University Hospital Solna, 17176 Stockholm, Sweden
b Clinical Epidemiology Unit, Department of Medicine, Karolinska Institute, Karolinska University Hospital Solna, Stockholm, Sweden
c Clinical Research Centre, Örebro University Hospital, Örebro, Sweden
d Nyamisati Malaria Research, Rufiji, National Institute for Medical Research, Tanzania

Received 12 June 2006; accepted 25 October 2006
Available online 6 December 2006

Abstract

Protective immunity to malaria is acquired after repeated exposure to the polymorphic Plasmodium falciparum parasite. Whether the number of concurrent antigenically diverse clones in asymptomatic infections predicts the risk of subsequent clinical malaria needs further understanding. We assessed the diversity of P. falciparum infections by merozoite surface protein 2 genotyping in a longitudinal population based study in Tanzania. The number of clones was highest in children 6–10 years and in individuals with long time to previous anti-malarial treatment. Individual exposure, analysed by circumsporozoite protein antibody levels, was associated with parasite prevalence but not with the number of clones. The risk of subsequent clinical malaria in children free of acute disease or recent treatment was, compared to one clone, reduced in individuals with multiclonal infections or without detectable parasites, with the lowest hazard ratio 0.28 (95% confidence interval 0.10–0.78 Cox regression) for 2–3 clones. The number of clones was not associated with haemoglobin levels. A reduced risk of malaria in asymptomatic individuals with multiclonal persistent P. falciparum infections suggests that controlled maintenance of diverse infections is important for clinical protection in continuously exposed individuals, and needs to be considered in the design and evaluation of new malaria control strategies.

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Keywords: Malaria; Plasmodium falciparum; Msp2; Diversity; CSP; Haemoglobin; Morbidity; Tanzania

1. Introduction

In areas of high Plasmodium falciparum transmission, individuals are repeatedly exposed to diverse infections and gradually acquire immunity which prevents clinical symptoms and high parasite burdens. Although the mechanisms for malaria immunity are still largely unknown, antigen specific responses are believed to be essential. The level of antigenic diversity to which the host is exposed to is thus important for the acquisition of protective immunity. The main aim of this study was to establish if the number of clones in asymptomatic P. falciparum infections is a marker of immune status and how the diversity predicts the host’s risk of subsequent clinical malaria.

The number of P. falciparum clones per infection, characterised by genetic polymorphisms in blood-stage antigens, varies with transmission intensity [1,2], age [1,3] and host genetics [4]. Within a small cohort of children in an area of high transmission in Tanzania, we described intra-individual consistency, over several years, in the number of infecting P. falciparum clones, as well as a trend of reduced morbidity in children with multiclonal infections [5]. A few studies with varying study designs, populations, age groups and transmission, have now reported associations both with reduced and increased risk of malaria [6–10].
We here present a study which provides evidence that asymptomatic multiclonal infections are associated with protection to clinical malaria. In contrast to previous studies, we undertook a strict definition of asymptomatic status, i.e. identified individuals free of acute or recent malaria to not bias the results by anti-malarial treatment or ongoing disease. The assessment of diversity of \textit{P. falciparum} parasite populations in the longitudinally followed population in a Tanzanian village included identification of factors which may influence the number of clones e.g. time to previous anti-malarial treatment and level of individual exposure. The genetic diversity of \textit{P. falciparum} infections was investigated by genotyping one of the major vaccine candidate antigens: the \textit{merozoite surface protein 2} (\textit{msp2}) \cite{11}. This highly polymorphic single copy gene is suitable for parasite population structure analysis since it remains stable in the haploid phase in the human host. Malaria morbidity was assessed in children and included both risk of febrile malaria and anaemia. The results suggest that the higher number of \textit{P. falciparum} \textit{msp2} genotypes, referred to as clones, in individuals with low risk of malaria disease does not only reflect previous exposure but also that antigenically diverse parasites \textit{per se} may contribute to maintenance of clinical protection in continuously exposed individuals.

2. Materials and methods

2.1. Study area and population

Nyamisati village is situated in Rufiji District, coastal Tanzania. Malaria transmission is perennial with some seasonal fluctuations. Previous parasite prevalence of $>75\%$ in children 2–9 years suggests a holoendemic setting \cite{12}. A research team, also providing health care, has lived in the village since 1985 and followed the population of about 1000 individuals with continuous monitoring of malaria. In March–April 1999, before the rainy period, 890 villagers 1–84 years participated in a cross-sectional survey including collection of venous blood and assessment of clinical status. Some 873 individuals had complete data sets and available DNA samples. The project was approved by the National Institute for Medical Research in Tanzania and the Ethical Committee at Karolinska Institute (Dnr 00-084).

2.2. Clinical data

Malaria episodes were continuously recorded through a passive case detection system (1993–1999) in which the villagers report to the unit in the event of fever, for diagnosis of malaria with microscopy, and free treatment. All individuals with fever and \textit{Plasmodium} parasites were treated with sulphadoxine—pyrimethamine (SP). In this study, the primary definition of a clinical malaria episode was fever (axillary temperature $>37.5\, ^\circ\text{C}$), reported or confirmed during the last day, and presence of \textit{P. falciparum} by microscopy. A second definition was fever together with $>5000$ parasites/µl blood, identified as specific definition for malaria in areas with high background parasite prevalence \cite{13}.

To clearly define the clinical status at the time of the survey i.e. to identify the truly asymptomatic individuals, those with fever (with or without parasites) ongoing or 1 week after the survey were excluded. Due to the prophylactic effect of SP \cite{14}, individuals treated within 4 weeks before the survey were also excluded from the asymptomatic group.

2.3. Sample collection

All samples were obtained after informed consent from the participants and/or their guardians. Venous blood was collected in EDTA tubes and stored frozen as plasma and packed cells. Haemoglobin levels were measured using a HemoCue photometer (Angelholm, Sweden).

2.4. Detection, enumeration and genotyping of \textit{P. falciparum} infections

Parasite densities were enumerated in 200 fields of Giemsa stained thick smears by conventional light microscopy (corresponding to 0.2 µl of blood). Genomic DNA, purified by phenol—chloroform extraction, was analysed by PCR of the \textit{msp2} \textit{P. falciparum} gene \cite{11}. A first amplification of the outer region of \textit{msp2} is followed by two separate nested reactions with allelic type-specific oligonucleotide primers (FC27 and 3D7/IC types). Length polymorphism of the PCR products was detected by electrophoresis on 2\% MetaPhor agarose gels (BMA, Rockland) and visualised by UV transillumination in Gel Doc 2000 (BioRad, CA) after ethidium bromide staining. The total number of \textit{msp2} alleles of the two types determined the number of clones per infection.

2.5. Antibody levels

Analysis of antibodies against circumsporozoite surface protein (CSP) of \textit{P. falciparum} was performed in a subset of 662 plasma samples (restricted by the amount of peptide, excluding only adults) using the synthetic (NANP)$_6$ peptide \cite{15}. Briefly, 96-well ELISA plates (Costar Corporation, USA) were coated with 50 µl of (NANP)$_6$ (10 µg/ml) overnight at 4 °C and saturated with 0.5\% BSA for 3 h at 37 °C. Plasma samples (1:1000) were incubated at 37 °C for 1 h, washed four times, and then incubated with the secondary antibody, goat anti-human IgG conjugated to alkaline phosphatase (1:1000) again at 37 °C for 1 h. The secondary antibody was incubated with \textit{p}-nitrophenylphosphate (Sigma—Aldrich, USA) for 1 h at room temperature. Optical densities (OD) were determined at 405 nm in a Multiskan EX reader (Labsystems, Helsinki, Finland), and after transformation antibody levels were expressed as µg/ml. Sera from African donors with high antibody levels and sera from unexposed Swedish donors were used as positive and negative controls, respectively. Determination of total IgE and anti-\textit{P. falciparum} IgG and IgE antibody levels by ELISA has been previously described for this survey \cite{16}.
2.6. Statistical analysis

Data analysis was performed using Statistica and SAS (version 8.0) software. The number of clones was categorised (0 negative PCR, 1, 2–3 and ≥4 clones), to avoid assumptions about linearity. Time to previous clinical malaria with sulphadoxine–pyrimethamine treatment was grouped into three categories: ≤20, 21–40 and ≥41 weeks. The relationship between parasite densities (grouped into quartiles of density distribution in microscopy positive) and the number of clones was tested by the Kruskal–Wallis test. Antibody levels were log-transformed for normal distribution. Multiple regression analysis was used to assess CSP-antibody- and haemoglobin levels, respectively, as dependent variables against *P. falciparum* prevalence, number of clones, total IgE, malaria-specific IgE and IgG and incidence of clinical malaria episodes before and after the survey. Age was grouped into five categories (<3, 3–5, 6–10, 11–16 and >16 years). Analyses including malaria morbidity were, due to rare events in adults, only performed in children. Risk of clinical malaria during 40 weeks follow-up was assessed in asymptomatic children (<16 years) as time to first event by Cox regression adjusting for age, sex, number of clones and previous clinical malaria, using both clinical definitions.

3. Results

3.1. Basic malariometric description

The prevalence of *P. falciparum* infections was 27% by microscopy and 46% by PCR in the 873 individuals (1–84 years, median 17 years), with highest prevalence of 74% in children 3–5 years, decreasing to 34% in adults. Twenty microscopy positive samples were, for unknown reasons negative by PCR despite repeated analysis. Of the 111 individuals with recorded or reported fever at the cross-sectional survey, 108 individuals (age 1–57, median 6 years) fulfilled the definition of clinical malaria, i.e. fever together with *P. falciparum*; of which 56 had >5000 parasites/μl and thus fulfilled the second clinical definition. Three individuals had fever without parasites. Six participants, of which three were parasite positive at the survey, had a history of SP treatment 4 weeks before the survey. These nine individuals were excluded from further analysis. No malaria episode was reported the week after the survey. In the 756, by our definition, strictly asymptomatic individuals, *P. falciparum* prevalence was 14% by microscopy and 38% by PCR, and was highest in children <3 years (58%). The median parasite densities were 800 parasites/μl (range 160–4960) in asymptomatic individuals, and 4800 parasites/μl (range 1600–152,000) and 18,400 parasites/μl (range 5600–152,000) in individuals symptomatic by the two respective definitions.

The number of malaria episodes, 40 weeks before and after the survey, were most frequent in children 3–5 years with 1.5 episodes/person year and decreased to 0.08 episodes/person year in individuals >16 years and were similar for both clinical definitions.

3.2. Msp2 genotyping: number of clones

Multiclonal *P. falciparum*, i.e. infections composed of ≥2 msp2 alleles, were detected in 280 (70%) of the 399 PCR positive samples. The mean number of msp2 alleles within the positive samples was 2.7 (range 1–7), with a mean of 2.4 (95% CI, 2.2–2.6) in the asymptomatic individuals. In the 108 individuals with fever and *P. falciparum*, the mean number of msp2 alleles was 3.4 (95% CI, 3.0–3.7), and 3.6 (95% CI, 3.1–4.0) in those with >5000 parasites/μl. The number of msp2 alleles was positively correlated with parasite densities (r = 0.296, P = 0.027), but such correlation was not found in the asymptomatic and symptomatic groups, respectively. The mean number of msp2 alleles in asymptomatic individuals was highest in children 6–10 years (mean 3.0) (Fig. 1A) whereas it was relatively constant over age in individuals with fever (Fig. 1B).

Of the two dimorphic types of msp2, the 3D7/IC type alleles were most prevalent, with 42% 3D7/IC, 14% FC27 and 44% with both types in asymptomatic individuals. In individuals with parasites and fever, 30% of infections were 3D7/IC, 6% FC27, and 64% contained both allelic types. The age profiles of the two msp2 allelic types differed in asymptomatic and symptomatic individuals, with more FC27 types in adults with fever (Fig. 1A and B).

- **Fig. 1.** Number of msp2 genotypes (mean ± SE) by age. (A) Asymptomatic individuals (□) (n = 288); (B) individuals with fever and *P. falciparum* (■) (n = 108). (□) Total msp2 genotypes; (◇) FC27; (△) 3D7/IC. PCR-negative samples were excluded.
3.3. Association between the number of clones and individual exposure — CSP (NANP)₆ antibody levels

Levels of antibodies against CSP (NANP)₆ in 662 samples (age 1–84, median 16 years) were stable up to 10 years of age followed by an increase in teenagers and adults (Table 1). Only including the asymptomatic individuals, children 1–10 years with *P. falciparum* had higher anti-CSP-antibody levels than children without parasites, a difference not found in the older individuals. Univariate analysis indicated an association between anti-CSP (NANP)₆ levels and age, number of malaria episodes per year, anti-*P. falciparum* IgE and IgG levels, number of clones and haemoglobin level. However, in a multiple regression analysis levels of anti-CSP (NANP)₆ antibodies are only significantly associated with anti-*P. falciparum* IgG (*P < 0.001*). After multiple adjustments, there was thus no association between anti-CSP levels and the number of clones (*P = 0.289*), clinical status at survey, nor subsequent risk of malaria, independent of clinical definition.

3.4. Association between the number of clones and previous anti-malarial treatment

Since malaria was rare in adults, assessment of morbidity were performed in young individuals (≤16 years, *n = 320*). Although, the parasite prevalence was not markedly affected by time to previous sulphadoxine–pyrimethamine treatment, multiple clones were more frequent in children without any clinical episode 40 weeks before the survey (Fig. 2). Compared to children with episodes within 20 weeks, children with no episodes had significantly lower frequency of single clone infections (*P = 0.025*, $\chi^2$ test) and higher frequency of multiclonal infections (*P = 0.017*). In the longitudinal records (1993–1999), all children had experienced clinical malaria before, confirming that all had been exposed.

3.5. Association between the number of clones and subsequent clinical malaria

The prospective risk of a clinical episode of malaria was based on time to the first episode of fever and parasites during 40 weeks follow-up in 320 children (≤16 years) who were asymptomatic at the survey. Hazard ratios (HR) for a subsequent malaria episode assessed by Cox regression analysis are presented in Table 2, including age, sex, number of clones and history of malaria episodes 40 weeks prior to the survey as individual baseline characteristics. The risk of subsequent malaria decreased with age, independent of the other measures, and was significantly higher in subjects with history of malaria, HR (adjusted) 2.43 (95% CI, 1.34–4.43). Compared with one clone, infections with ≥2 clones as well as absence of detectable parasites were associated with reduced risk of subsequent malarial disease (Fig. 3). After simultaneous multiple adjustments for individual factors, HR were 0.28 (95% CI, 0.10–0.78) for 2–3 genotypes, 0.42 (95% CI, 0.15–1.17) for ≥4 clones and 0.53 (95% CI, 0.29–0.96) in subjects with no parasites. Similar results were also obtained for both clinical definitions.

3.6. Associations between number of parasite genotypes and haemoglobin levels

Haemoglobin (Hb) levels increased with age (*P < 0.001*) (Table 3; figure not shown). In children 1–10 years, the mean Hb values were lower in symptomatic (96 g/l, 95% CI, 93–100) compared to asymptomatic individuals (104 g/l, 95% CI, 102–107) (*P = 0.001*), respectively. Seventeen individuals, six with fever and parasites at the survey, fulfilled the WHO criteria of severe to moderate anaemia (Hb < 70 g/l), and one the criteria of severe anaemia (Hb < 50 g/l). In individuals with fever, Hb levels decreased with increasing parasite densities ($r = -0.413$, $P = 0.022$) but not with the number of clones. In asymptomatic individuals Hb values were higher in those with long time to a previous clinical episode.
4. Discussion

In a previous small study we observed a trend that asymptomatic children with continuous multiclonal *P. falciparum* infections were at a reduced risk of malaria compared to children with single clone infections [5]. The current more in depth population based assessment confirms that multiclonal infections in asymptomatic children are associated with greater protection against clinical malaria in this high transmission setting. Individuals with efficient protective immunity may be expected to harbour fewer *P. falciparum* clones compared to more susceptible individuals of the same age. In contrast, our findings reveal that the diversity of parasite populations is an important component of host–parasite interactions which contribute to protective malaria immunity in continuously exposed individuals.

The present study included an assessment of different factors that may affect the number of clones in an individual at a certain time (clinical status, time to previous anti-malarial treatment, exposure). Since febrile infections had higher diversity, we decided to exclude individuals with ongoing clinical malaria in the risk assessment. Moreover, considering the prophylactic effect of sulphadoxine–pyrimethamine [14], individuals with anti-malarial treatment within 4 weeks prior to the survey were also excluded. We considered this to be a conservative approach to assess the prospective disease risk in asymptomatic individuals.

Previous studies relating *P. falciparum* diversity and morbidity have come to different conclusions. A couple of initial studies suggested a protective association with multiclonal infections [5–7]. The follow-up studies have, however, shown an increased risk [8,17,18] mainly in small children. The low number of infants in this study restricted specific analysis in this age group. Discrepancies between studies, although in some not clearly significant results, may partly be explained by differences in transmission levels, ages and herd immunity but also by study designs and statistical approaches. We believe that our strict definition of asymptomatic status contributed to a more distinct assessment. Indeed when the symptomatic individuals were also included in the analysis the protective associations were not apparent (data not shown).

Table 2
Prospective risk of clinical malaria episode in asymptomatic children (≤16 years) assessed by Cox regression analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>Subsequent episodea</th>
<th>HRb unadjusted (95% CI)</th>
<th>HR adjustedc (95% CI)</th>
<th>HR adjustedd (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3 years</td>
<td>22</td>
<td>11 (50%)</td>
<td>1.00*</td>
<td>1.00*</td>
<td>1.00*</td>
</tr>
<tr>
<td>3–5 years</td>
<td>61</td>
<td>18 (30%)</td>
<td>0.50 (0.24–1.06)</td>
<td>0.55 (0.26–1.17)</td>
<td>0.60 (0.28–1.30)</td>
</tr>
<tr>
<td>6–10 years</td>
<td>112</td>
<td>17 (15%)</td>
<td>0.23 (0.11–0.49)</td>
<td>0.27 (0.12–0.58)</td>
<td>0.35 (0.16–0.78)</td>
</tr>
<tr>
<td>11–16 years</td>
<td>125</td>
<td>12 (10%)</td>
<td>0.14 (0.06–0.32)</td>
<td>0.17 (0.07–0.38)</td>
<td>0.28 (0.11–0.70)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>146</td>
<td>25 (17%)</td>
<td>1.00*</td>
<td>1.00*</td>
<td>1.00*</td>
</tr>
<tr>
<td>Male</td>
<td>174</td>
<td>33 (19%)</td>
<td>1.09 (0.65–1.83)</td>
<td>1.12 (0.66–1.89)</td>
<td>1.01 (0.59–1.71)</td>
</tr>
<tr>
<td>Msp2 genotypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>157</td>
<td>29 (18%)</td>
<td>0.47 (0.26–0.83)</td>
<td>0.49 (0.27–0.88)</td>
<td>0.53 (0.29–0.96)</td>
</tr>
<tr>
<td>1</td>
<td>54</td>
<td>19 (35%)</td>
<td>1.00*</td>
<td>1.00*</td>
<td>1.00*</td>
</tr>
<tr>
<td>2–3</td>
<td>62</td>
<td>5 (8%)</td>
<td>0.20 (0.07–0.52)</td>
<td>0.24 (0.09–0.65)</td>
<td>0.28 (0.10–0.78)</td>
</tr>
<tr>
<td>≥4</td>
<td>47</td>
<td>5 (11%)</td>
<td>0.26 (0.10–0.69)</td>
<td>0.34 (0.13–0.94)</td>
<td>0.42 (0.15–1.17)</td>
</tr>
<tr>
<td>Malaria history</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>224</td>
<td>24 (11%)</td>
<td>1.00*</td>
<td>–</td>
<td>1.00*</td>
</tr>
<tr>
<td>Yes</td>
<td>96</td>
<td>34 (35%)</td>
<td>3.83 (2.27–6.47)</td>
<td>–</td>
<td>2.43 (1.34–4.43)</td>
</tr>
<tr>
<td>Total</td>
<td>320</td>
<td>58</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

a Reference group.

b HR, hazard ratio.

c Hazard ratios adjusted for age, sex and number of clones.

d Hazard ratios adjusted for age, sex, number of clones, and history of clinical malaria 40 weeks before.
The lowest risk of subsequent malaria was found in asymptomatic children with 2–3 clones with hazard ratio 0.28 (95% CI, 0.11–0.70) (Table 2), compared to those with one clone. A reduced risk was, however, also found in individuals without detectable parasites. In low endemic areas parasite negativity may reflect a lack of exposure. Here, all villagers are expected to be repeatedly infected and absence of detectable parasites rather indicates efficient anti-parasitic immunity. Considering the non-sterilising nature of anti-malarial immunity, also PCR-negative individuals may indeed harbour parasites at levels below detection. The highest risk was found in children with only one clone, suggesting that these individuals are not as capable of managing the challenge of additional clones without developing disease compared to those with multiclonal infections who apparently remain asymptomatic despite several concurrent P. falciparum clones. There was, however, no dose–response between the number of clones and protection. Compared to one clone, 2–3 clones were associated with a reduced risk but more clones were not even more protective (Table 2). We chose to use a non-linear approach when assessing the number of clones and parasite negativity based on previous findings that immune responses to malaria may not be linear [16]. A linear model would indeed not have detected these optimal levels of parasite population diversity. The findings suggest that protective anti-malarial immunity is based on equilibrium and controlled persistence of parasite populations, and that different elements of immune function may regulate clearance of parasite loads. This state of immunological balance is also supported by the intra-individual consistency in number of P. falciparum clones in relation to morbidity in our previous study [5].

Among factors that may influence diversity, time to previous treatment was significantly associated with the number of clones. Although parasite prevalence was relatively constant, the diversity was higher in individuals with long time to previous treatment (Fig. 2). This suggests that parasite populations are accumulated with time in partly immune individuals but may also reflect less frequent clinical episodes in these individuals. When adjustments were made for time to previous malaria episode, multiclonal infections were still associated with a reduced prospective risk of disease (Table 2). If prior infections are part of the causal process, this approach may be an over-adjustment producing conservative estimates. Moreover excluding children with ongoing or recent episode of clinical malaria, may also have underestimated associations, since the most susceptible individuals were not analysed. Adjustments for prior episodes thus support that the presence of multiclonal parasites per se is involved in protection. Clinical studies as well as experimental models have indeed suggested a significant impact of chronic parasite carriage on development of clinical immunity [19].

The host’s interaction with different levels of parasite polymorphisms is reflected by the age profiles of the number of clones in different settings. In low endemic areas, the number of clones is low and rather constant over age [1] whereas in high endemic areas the highest number of clones per infection is found in school age children. Here, the highest diversity was found in children 6–10 years, which is concordant with reports from high transmission areas [1,9]. The level of diversity

Table 3
Haemoglobin levels in relation P. falciparum prevalence, number of clones and clinical status in different age groups (n = 864)

<table>
<thead>
<tr>
<th>Number of clones</th>
<th>1–10 Years</th>
<th>11–16 Years</th>
<th>≥17 Years</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean± (95% CI)</td>
<td>P&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>P. falciparum&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Negative</td>
<td>96</td>
<td>106 (103–109)</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>99</td>
<td>102 (98–107)</td>
</tr>
<tr>
<td>Number of clones&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
<td>37</td>
<td>101 (95–107)</td>
</tr>
<tr>
<td></td>
<td>2–3</td>
<td>33</td>
<td>105 (96–114)</td>
</tr>
<tr>
<td></td>
<td>≥4</td>
<td>29</td>
<td>101 (95–108)</td>
</tr>
<tr>
<td>Clinical status&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Asymptomatic</td>
<td>195</td>
<td>104 (102–107)</td>
</tr>
<tr>
<td></td>
<td>Fever + Pf</td>
<td>80</td>
<td>96 (93–100)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Reference group.
<sup>b</sup>Haemoglobin levels (g/l), mean value.
<sup>c</sup>Presence of P. falciparum by PCR in asymptomatic individuals.
<sup>d</sup>Number of clones determined by msp2 genotyping in asymptomatic individuals with P. falciparum.
<sup>e</sup>Clinical health status at the survey, asymptomatic vs. with symptoms of clinical malaria.
<sup>P</sup>-value, by t-test for independent samples.
with a mean of 2.7 msp2 genotypes was, however, lower than expected. This may be explained by the fact that the survey was performed before the rainy season, which affects parasite prevalence [12]. Moreover, we suspect that establishment of a health clinic with continuous monitoring of malaria and provision of early treatment may have changed parasite prevalence. The spleen rates have decreased from >75% in 2–9 year old children [12] to 34% in 1993 which rather indicates hyper- to mesoendemic malaria. Spleen size was not assessed in this survey but also the parasite prevalence in the same age group decreased from >75% in 1986 to <50% in 1999.

The association between multiclonal infections and protection may reflect a higher level of previous exposure in protected individuals. Antibodies against circumsporozoite protein (CSP), considered the best available serological marker of exposure [20,21], were higher in children with parasites, but were not associated with the number of clones (Table 1), confirming findings in a recent report [22], and suggesting that exposure may not be the only factor which affects the diversity of P. falciparum infections. Here anti-CSP levels followed previous age profiles [23] and correlated to anti-P. falciparum (crude) specific IgG levels previously measured [16], but in contrast to anti-P. falciparum (crude) specific IgE levels [16], none of these two antibodies were associated with risk of subsequent malaria.

The P. falciparum parasite expresses many antigens to which antibodies are detected in populations in endemic areas. The extensive diversity within many of these antigens, including the merozoite surface proteins, stimulates highly specific antibody responses [24,25]. There is also growing evidence for the importance of antibodies to the variant surface antigen (VSA) P. falciparum erythrocyte membrane protein 1, a molecule coded by multiple genes that undergoes switching during (VSA) for the importance of antibodies to the variant surface antigen P. falciparum of clones and concurrent anaemia. An association was, however, not found between the number of clones and concurrent anaemia. As a result, one may hypothesise that clearance of asymptomatic multiclonal parasitemia may influence the subsequent risk of clinical malaria, which may possibly argue against a generalized use of chemophrophylaxis or intermittent preventive treatment in children older than 1 year in areas with high malaria endemicity. The concept of controlled equilibrium between multiclonality, parasite clearance and protection needs to be integrated in the understanding of acquired immunity as well as in the development of new malaria control interventions.

Acknowledgements

We are most grateful to the villagers and the research team in Nyamisati, who participated in this study. We thank Danielle Carpenter, Marie Anne Shaw, Margareta Hagstedt, Marita Troye-Blomberg and Zul Premji for most valuable expertise and technical assistance. Financial support was received from Swedish International Development Cooperation Agency-SAREC (Project grant SWE 2002-066) and Sigurd and Elsa Goljes Foundation.

References


