INSTITUT PASTEUR

Microbes and Infection 9 (2007) 103–110

www.elsevier.com/locate/micinf

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.

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].

* Corresponding author. Tel.: +46 851 775285; fax: +46 851 771806.
E-mail address: anna.farnert@ki.se (A. Färnert).

1286-4579/$ - see front matter © 2006 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.micinf.2006.10.014

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 *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 *P. falciparum* infections was investigated by genotyping one of the major vaccine candidate antigens: the *merozoite surface protein 2* (*msp2*) [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 *P. falciparum 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 *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 [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 *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 °C), reported or confirmed during the last day, and presence of *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 [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 [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 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 *msp2 P. falciparum* gene [11]. A first amplification of the outer region of *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 *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 *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 [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 *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-*P. falciparum* IgG and IgE antibody levels by ELISA has been previously described for this survey [16].