Epidemiology of multiple *Plasmodium falciparum* infections

6. Multiple *Plasmodium falciparum* infections in Tanzanian infants

I. Felger[1]*, T. Smith[1], D. Edoh[2]†, A. Kitua[2], P. Alonso[3], M. Tanner[1] and H.-P. Beck[1]** *[1]Swiss Tropical Institute, Socinstrasse 57, 4002 Basel, Switzerland; [2]Hospital Clinic i Provincial, Barcelona, Spain; [3]Ifakara Health Research and Development Centre, P.O. Box 53, Ifakara, Tanzania*

Abstract

Paired blood samples from 99 Tanzanian infants were analysed to examine the infection dynamics of *Plasmodium falciparum* during the first year of life. Infecting parasites were genotyped by polymerase chain reaction amplification of the polymorphic gene for the merozoite surface protein 2 and subsequent analysis according to the resulting restriction fragment length polymorphism pattern. The same samples served as controls in a parallel case–control study for which an additional blood sample was taken from each child during a fever episode. The relationship of the number of concurrent infections (multiplicity) with age and morbidity was analysed and results were compared to those of a similar study on older children between 2 and 7 years of age, carried out in the same village at the same time. The mean of 2 infecting genotypes per positive blood sample from community surveys was low compared to that in older children, and there was no significant age-dependency of multiplicity within the first year of life. Multiplicity of infection in fever cases was also independent of age. In infants, multiplicity was positively associated with parasite density and risk of clinical malaria, in contrast to the situation in older children (>2 years). The findings help in the understanding of infection dynamics, premunition, and development of semi-immunity in malaria.

Keywords: malaria, *Plasmodium falciparum*, multiple infection, genotypes, *msp2* gene, morbidity, premunition, immunity, Tanzania

Introduction

Infants rarely fall ill with malaria in their first months of life. Very young infants in areas holoendemic for malaria show mild symptoms when infected, and they are comparable to partially immune adults with respect to parasite densities and recovery rates from infection (KITUA *et al.*, 1996). This protection during the first 1–3 months of life is thought to be conveyed by fetal haemoglobin (PASVOL *et al.*, 1977) or maternal malaria-specific immunoglobulin G antibodies, which can be detected in cord blood and are acquired across the placenta (DESOWITZ *et al.*, 1993; RASHEED *et al.*, 1995). Parasite prevalence and density increase steeply for the first 6 months (ACHIDI *et al.*, 1996; KITUA *et al.*, 1996). In a study of Tanzanian infants, the incidence rates of malaria-attributed morbidity reached a peak at 6 months of age (KITUA *et al.*, 1996). As children grow older they experience fewer malaria episodes until a state of semi-immunity is reached at adolescence. Thus, the period of highest susceptibility to clinical malaria among infants in such highly endemic areas occurs between the ages of 4 and 12 months.

Plasmodium falciparum isolates from areas of high transmission were found to consist of multiple concurrent infections (CONTAMIN *et al.*, 1995). Mean multiplicity in children was 4 infections per carrier in Senegal (NTOUMI *et al.*, 1995) and 5 infections per asymptomatic carrier in Tanzania (BECK *et al.*, 1997). All blood samples characterized so far were obtained from children over one year of age. In infants, however, little is known about the infection dynamics of individual *P. falciparum* clones, and no information is available on multiplicity of infection, the acquisition and loss of multiple infections, or their association with morbidity. It can be expected that multiplicity in infants differs considerably from that in older children, as does prevalence and parasite density (KITUA *et al.*, 1996). Initial, but waning, maternal protection and subsequent slow acquisition of immune competence are likely to affect the infection dynamics in the first year of life.

To address these questions, we genotyped the infecting parasites from consecutive blood samples from Tanzanian infants under one year of age, from an area where transmission of malaria is intense and perennial with more than 300 infectious bites per year (SMITH *et al.*, 1993). Community samples from mostly asymptomatic individuals were used to analyse the acquisition and distribution of individual infections during the first year of life, and to test whether the accumulation of multiple infections was age-dependent. The genetic diversity of *P. falciparum* parasites infecting these children was examined using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis of the highly polymorphic parasite gene for the merozoite surface protein 2 (*msp2*) (FELGER *et al.*, 1993, 1994). In addition, a matched case–control study including the same children was conducted in order to test for associations of specific genotypes with morbidity, and to estimate the risk of fever with respect to multiple infections in the first year of life. Previous studies on older children have shown that concurrent multiple infections are associated with protection against clinical malaria in endemic areas (AL-YAMAN *et al.*, 1997; BECK *et al.*, 1997). Certain genotypes have been associated with higher parasite densities and morbidity (ENGELBRECHT *et al.*, 1995). In the present study we were interested in finding out whether this protection by multiple infections already operates in infants.

Materials and Methods

Sampling

The study was conducted in Idete, a village in the Kilombero valley of Tanzania. The area is holoendemic for malaria, with perennial transmission and little seasonality of parasite prevalence and density (SMITH *et al.*, 1993). The study area and overall study design have been described elsewhere (KITUA *et al.*, 1996). Children included in the present substudy were an age-stratified random sample of all children from Idete under the age of 12 months. The finger-prick blood samples analysed were collected during household visits carried out from November 1993 to July 1994. Thick and thin films for microscopical examination were made from all blood samples as described earlier (KITUA *et al.*, 1996).

*Author for correspondence: phone +41 61 284 8116, fax +41 61 271 8654, e-mail <felger@ubaclu.unibas.ch>
†Present address: Department of Zoology, University of Ghana, Box 67, Legon, Accra, Ghana.
**Present address National Institute for Medical Research. P.O. Box 9635, Dar es Salaam, Tanzania.

For the present study, 2 samples, A and B, collected approximately one month apart, were chosen from each of 99 infants (no. of samples=198), regardless of parasitaemia. The times were chosen to generate approximately equal numbers of samples for each month. In order to conduct an additional matched case–control study, a third sample (C) from each child was analysed (if available) from a febrile episode (axillary temperature ≥37·5°C, regardless of parasitaemia) reported at the village dispensary either at least 4 weeks before samples A and B were collected, or at least 4 weeks after. Such samples from a febrile episode were available for 62 of the 99 children. For the calculation of risk of clinical malaria, all 53 parasite-positive samples from febrile children collected at the dispensary and all 13 parasite-positive samples from febrile children collected during the community surveys were included. Neither multiplicity of infection (Wilcoxon's Z=0·09, P=0·9) nor parasite density (Wilcoxon's Z=0·13, P=0·9) was significantly different between both sets of samples, in contrast to previous findings by COX *et al.* (1994).

Genotyping

Deoxyribonucleic acid (DNA) from all blood samples, irrespective of their microscopically determined parasitaemia, was subjected to primary and nested PCR amplification using *msp2* specific primary and nested primer pairs. Identification of *msp2* alleles was done by RFLP analysis. Conditions of PCR amplification and the PCR–RFLP genotyping technique used are described in detail elsewhere (FELGER *et al.*, 1999).

Statistical analysis

Allele frequencies were calculated by the method of HILL & BABIKER (1995), assuming a negative binomial distribution for the total distinct genotypes seen in any one child.

The null hypothesis that *msp2* genotypes do not vary in their association with morbidity was tested using a 2×50 contingency table containing the numbers of occurrences of each allele separately in asymptomatic and sick individuals. The Pearson goodness-of-fit statistic was computed to test the hypothesis of a constant ratio between occurrences in sick children and occurrences in asymptomatic children.

The usual χ^2 test for significance of the Pearson statistic is conservative, owing to the data being sparse. To allow for this, the null distribution of the test statistic was estimated from random allocations of genotypes between cases and controls. A corrected value of P was then computed from the proportion of randomly generated data sets which gave higher goodness of fit statistics than that found in the actual data set.

Results

Sample numbers, parasite positivity rate and fever incidence as inclusion criteria for the case–control analysis are summarized in Table 1. The distribution of all

Table 1. *Plasmodium falciparum* infection status as determined by polymerase chain reaction and fever status of children providing blood samples

	Samples	
	A+B[a]	C[b]
P. falciparum		
Negative	90	9
Positive, no fever[c]	95	0
Positive, fever[c]	13[d]	53[d]
Total	198	62

[a]Community survey samples collected one month apart.
[b]Samples from self-reported patients at the village dispensary at least 4 weeks before or after collection of samples A and B.
[c]Fever was defined as axillary temperature ≥37·5°C.
[d]These children were included as cases in the matched sets analysis for the relative risk of clinical malaria.

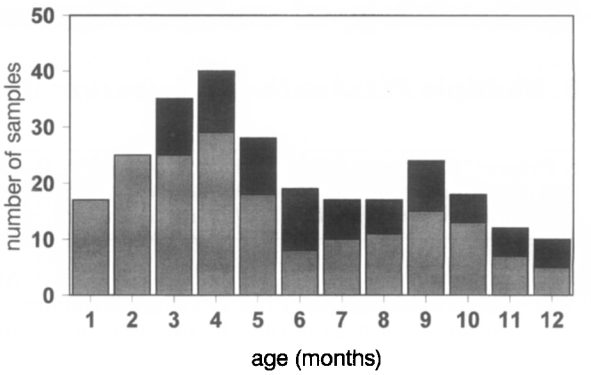

Fig. 1. Age distribution of the donors of 260 blood samples analysed for *msp2* genotypes of *Plasmodium falciparum*. Lightly shaded blocks represent asymptomatic children from community surveys, dark shaded blocks represent fever cases from the community or reported at the village dispensary.

Table 2. Frequencies of *msp2* genotypes in *Plasmodium falciparum* samples

	Community samples[a]		Asymptomatic infants[a]	Febrile infants[a,b]
Alleles	No. of infections	Allele frequency	No. of infections	No. of infections
Alleles of the 3D7 family[c]				
3D7(240)	–	–	–	1(0·6%)
3D7(250)	0	–	–	2(1·2%)
3D7(270)	0	–	–	2(1·2%)
3D7(280)	2	0·009	2(1·2%)	–
3D7(290)	–	–	–	3(1·7%)
3D7(300)	1	0·005	–	1(0·6%)
3D7(310)	2	0·014	3(1·8%)	–
3D7(320)	5	0·034	5 (3%)	3(1·7%)
3D7(330)	15	0·079	12(7·1%)	14(8·1%)
3D7(335)	1	0·005	1(0·6%)	–
3D7(340)	3	0·019	3(1·8%)	6(3·5%)
3D7(350)	5	0·024	5 (3%)	3(1·7%)
3D7(360)	10	0·044	5 (3%)	12 (7%)
3D7(370)	14	0·073	13(7·7%)	5(2·9%)
3D7(380)	4	0·019	4(2·4%)	2(1·2%)
3D7(390)	4	0·019	4(2·4%)	6(3·5%)
3D7(400)	7	0·034	7(4·2%)	5(2·9%)
3D7(410)	4	0·019	3(1·8%)	5(2·9%)
3D7(420)	5	0·024	4(2·4%)	6(3·5%)
3D7(430)	3	0·014	3(1·8%)	3(1·7%)
3D7(440)	5	0·024	4(2·4%)	3(1·7%)
3D7(450)	8	0·044	7(4·2%)	4(2·3%)
3D7(460)	3	0·014	3(1·8%)	–
3D7(470)	1	0·005	1(0·6%)	1(0·6%)
3D7(480)	2	0·009	2(1·2%)	–
Ifa6	2	0·009	2(1·2%)	–
Alleles of the FC27 family				
D10	4	0·019	4(2·4%)	3(1·7%)
K1	13	0·066	11(6·5%)	24 (14%)
Ifa1	–	–	–	1(0·6%)
Ifa13	1	0·005	1(0·6%)	1(0·6%)
Ifa17	1	0·005	–	1(0·6%)
Ifa18	1	0·005	1(0·6%)	–
Ifa27	1	0·005	1(0·6%)	1(0·6%)
Ifa28	–	–	–	1(0·6%)
Ifa30	1	0·005	–	1(0·6%)
Ifa31	–	–	–	2(1·2%)
Ifa32	–	–	–	1(0·6%)
Ifa33	–	–	–	1(0·6%)
Ifa34	2	0·009	2(1·2%)	1(0·6%)
Ifa42	1	0·005	1(0·6%)	–
Ifa43	4	0·019	4(2·4%)	1(0·6%)
Wos3	14	0·074	13(7·7%)	13(7·6%)
Wos6	5	0·024	5 (3%)	3(1·7%)
Wos7	12	0·062	9(5·4%)	9(5·2%)
Wos10	–	–	–	1(0·6%)
Wos12	23	0·140	19(11·3%)	19 (11%)
Wos34	2	0·009	2(1·2%)	–
Ifa44	–	–	–	1(0·6%)
Ifa45	1	0·005	1(0·6%)	–
Ifa23	1	0·005	1 (0·6%)	–
Total	193	1·000	168(100%)	172(100%)

[a]Community survey samples derived from both asymptomtic and febrile infants. The analysis of asymptomatic infants included all community samples except those from febrile cases; that of febrile infants included samples from community surveys and health centre cases as listed in Table 1.
[b]Axillary temperature ≥37·5°C.
[c]Numbers in parentheses indicate the length (bp) of the large *Hin*fI fragment after restriction digestion.

260 samples with respect to the blood donors' age at the midpoint between the sample intervals is shown in Fig. 1. Of the samples from community surveys, 44% were positive for *P. falciparum* by microscopy (asexual blood stages and gametocytes). The highest density found was 69000 parasites/μL of blood. By PCR, 54·5% of the blood samples were positive for *P. falciparum*. Thus, 22 microscopically negative samples produced a PCR product, which confirmed the higher sensitivity of parasite detection by PCR.

Parasite positivity rate and allele frequencies

A total of 193 distinct infections was identified in the 108 community samples containing *P. falciparum*. Of those, 28 infections occurred in both paired samples, 104 were absent from the first sample but present in the second, and 61 infections were present in the first sample but were not detectable by the time the second sample was taken. Details of the infection dynamics are presented in another paper (SMITH *et al.*, 1999b). In 66 PCR-positive fever cases from the case–control study, 172 individual infections were detected.

Overall, 50 different *msp2* alleles (Table 2) were found in samples from either asymptomatic or febrile infants. When these alleles were grouped into 2 allelic families according to the dimorphic structure of the central variable region (SMYTHE *et al.*, 1990), 24 alleles belonged to the FC27 family of alleles and 26 to the 3D7 family. Gene frequencies were calculated for genotypes deriving from the paired community samples. Alleles occurring in both community samples of the same individual were counted only once. The most frequent allele in the community samples was Wos12, a member of the FC27 family, which accounted for 12% of all infections detected. Wos3, Wos7 and K1 were further alleles of the same family with frequencies >0·05. The highest allele frequencies within the 3D7 family were found for $3D7_{370}$ and $3D7_{330}$, which accounted for 7% and 8% of all genotypes, respectively. All other 3D7-type genotypes showed frequencies <0·05 and 10 alleles were found only once.

Differences in distribution of *msp2* genotypes between asymptomatic and febrile infants are also summarized in Table 2. Ten genotypes were found only in asymptomatic individuals, all with low frequencies, and 13 genotypes were detected only in febrile individuals, also with low frequencies. The largest difference in allele frequencies between the 2 groups was seen between the number of K1 genotypes, found in 14% (24/172) of detected infections in febrile individuals and in 6·7% (11/168) of infections in asymptomatic individuals. Notable difference were also observed with $3D7_{360}$ and $3D7_{370}$, as shown in Table 2. However, although the frequencies of these genotypes appeared to differ substantially between asymptomatic and sick individuals, the Pearson goodness-of-fit test (null hypothesis: no difference in morbidity association between genotypes) indicated that the difference was not statistically significant, both when it was assessed using the conventional (conservative) significance level or when corrected for sparse data ($\chi_{49}^2=57{\cdot}4$, uncorrected $P=0{\cdot}19$; randomization test allowing for sparse data, $P=0{\cdot}15$). This suggested that the differences in frequency were probably chance fluctuations.

Age distribution of multiplicity of infection

Overall parasite prevalence determined by PCR increased with age. This was in agreement with the prevalence established by microscopy (KITUA *et al.*, 1996). The detected *msp2* alleles were grouped either in the 3D7 or FC27 allelic family, in order to test for differences in the age distribution of the 2 families, but no difference was detected.

The age distribution of multiplicity of infection during the first year of life was of great interest, because in older, asymptomatic Tanzanian children mean multiplicity had been found to be high, with 5 different concurrent infections (BECK *et al.*, 1997). Thus, it was to be expected that multiplicity in asymptomatic infants might slowly increase within the first year of life. Our results from the community survey, including mostly asymptomatic infants, revealed a rapid acquisition of infections after birth, but at the same time an unexpectedly low mean multiplicity of 2·1 infections per carrier. Multiplicity did not increase significantly with age during the first year of life (Spearman's $\rho=0{\cdot}11$, $P=0{\cdot}11$). Fig. 2 shows the age distribution of multiplicity of infections in PCR-positive samples at two-monthly intervals. When mean multiplicity was analysed separately for genotypes of both allelic families, again no age-dependency was found (FC27-type alleles; $\rho=0{\cdot}17$, $P=0{\cdot}089$; 3D7-type alleles: $\rho=0{\cdot}04$, $P=0{\cdot}7$). To reconcile these new data with the previous data from older children, a substantial increase in multiplicity of infection has to be assumed for the age period between one and 2 years. In this context it is noteworthy that from the age of 7 months onwards a suggestion of increase in multiplicity was evident (Fig. 2).

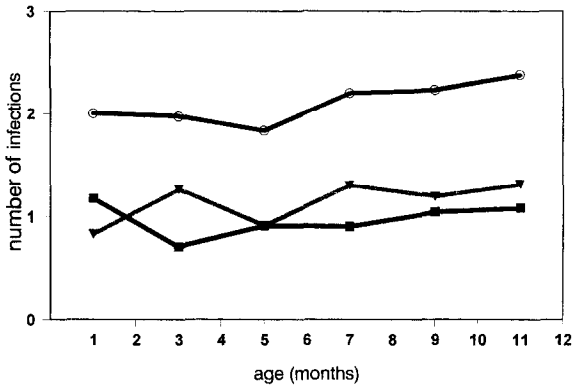

Fig. 2. Mean multiplicity of *Plasmodium falciparum* infections during the first year of life. Means are calculated from intervals of 2 months; all *msp2* genotypes (O), FC27-type genotypes (■) and 3D7-type (▼) genotypes are shown.

Table 3. Multiplicity of infection and parasite density in children infected with *Plasmodium falciparum* according to age group and genotype

	No. of samples		Multiplicity of infection[a]					
			All genotypes		3D7-like genotypes		FC27-like genotypes	
Age (months)	Asymptomatic children	Febrile children	Asymptomatic children	Febrile children	Asymptomatic children	Febrile children	Asymptomatic children	Febrile children
1–2	6	0	2·0(0·9	–	0·8(1·0)	–	1·2(1·2)	–
3–4	24	13	1·8(0·9)	2·2(1·5)	1·2(0·8)	1·2(1·1)	0·6(0·7)	1·0(0·7)
5–6	19	20	1·6(1·1)	3·0(1·4)	0·8(0·9)	1·6(1·3)	0·8(0·8)	1·4(0·9)
7–8	10	14	2·2(1·2)	2·5(1·8)	1·3(0·7)	1·1(1·1)	0·9(1·2)	1·4(1·1)
9–10	24	8	2·2(1·3)	3·1(2·6)	1·2(1·0)	1·8(2·0)	1·0(0·9)	1·4(1·1)
11–12	12	11	2·2(1·4)	2·7(1·5)	1·3(1·1)	1·2(0·4)	0·9(0·8)	1·5(1·2)
All	95	66	2·0(1·1)	2·7(1·7)	1·1(0·9)	1·4(1·2)	0·9(0·9)	1·3(1·0)

[a]Mean (SD in parentheses).

Multiplicity of infection with respect to fever and parasite densities

The multiplicity found in febrile and in asymptomatic children is shown in Table 3 for each age group separately and for both allelic families. In this case–control analysis, infants experiencing a febrile episode had a significantly higher mean multiplicity than asymptomatic infants (mean multiplicity of 2·7 infections versus 2·0 infections, χ^2=7·48, P<0·01). There was no difference between the allelic families with respect to their contribution to higher multiplicity in febrile infants. Multiplicity was independent of age both in asymptomatic community samples and in fever cases.

Analysis of the relationship between multiplicity and parasite density considered only PCR-positive cross-sectional samples. Samples which were positive by PCR but negative by microscopy were assigned a density of 10 parasites/μL of blood. Table 4 shows the geometric mean parasite density by multiplicity. There are clear trends of an increase in parasite density with increased multiplicity. Regression analysis of the log-transformed parasite density confirmed a statistically significant relationship between multiplicity and density ($F_{1,102}$=12·6, P<0·001). Each additional infection multiplied the average density by a factor of 1·9 (95% confidence interval 1·3–2·7). There was a statistically significant increase in parasite density with increasing number of 3D7-like genotypes ($F_{1,102}$=11·5, P=0·001). It appeared that density also increased with multiplicity of FC27-like genotypes, but the 6 samples containing 3 FC27-like genotypes each had, on average, low parasite densities and were not clustered in any particular age group. Therefore, no significant effect of the number of FC27-like genotypes on parasite density was found ($F_{1,102}$=1·9, P=0·17). There was no statistically significant interaction between age and multiplicity in their effect on density ($F_{1,101}$=0·11, P=0·7).

Conditional logistic regression models were used to analyse the relative risk of fever as a function of multiplicity. The risk of fever increased with the multiplicity of both FC27-like and 3D7-like genotypes (Fig. 3).

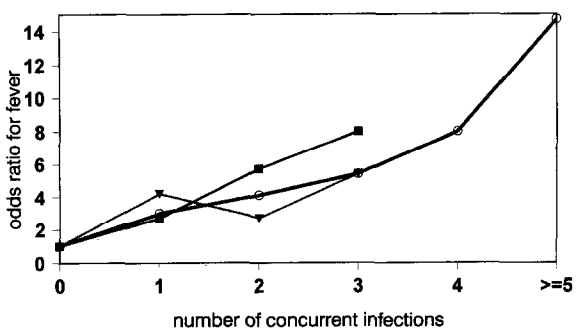

Fig. 3. Odds ratio for fever in relation to multiplicity of infection; all *msp2* genotypes (○), FC27-type genotypes (■) and 3D7-type genotypes (▼) are shown.

Discussion

Infants are immunologically naïve and therefore, when living in areas holoendemic for malaria, they suffer the highest numbers of malaria episodes, many of them being severe. In older children, the risk of clinical malaria has been shown to be correlated with multiplicity of infection (BECK *et al.*, 1997). Therefore, the acquisition of multiple infections and their relationship to morbidity may be of great importance in the development of natural immunity against *P. falciparum*. While all previous studies have suggested that high multiplicity of infection is protective against clinical malaria (ROBERT *et al.*, 1996; AL-YAMAN *et al.*, 1997; BECK *et al.*, 1997), in the present study (the only one focusing on the first year of life) the opposite result was obtained: multiplicity of infection was greater in fever cases than in asymptomatic parasite carriers.

This finding probably reflects the immune status of these very young children. In an early stage of the developing immune response against malaria, most new infections cause disease, as indicated by the two-fold increase in risk of fever with every additional infecting genotype. The same is suggested by the correlation between density and multiplicity of infection, showing that with every additional infection the average density was multiplied by a factor of about 2. Clinical malaria has antiparasitic effects due to non-specific immune responses, such as high fever and cytokine effects (KWIATKOWSKI, 1991), which might result in a reduction of parasite density. This could contribute to the elimination of other concurrently infecting parasite clones of low density, reducing the subsequent multiplicity of infection in the infants. We have investigated this possibility in further analyses of the dynamics of infection and parasite clearance in these infants (SMITH *et al.*, 1999b).

In older children, already possessing some clinical immunity, new infections do not always trigger this pyrogenic cytokine cascade (ROGIER *et al.*, 1996) and this reduction in the non-specific antiparasitic response could allow individual clones to persist longer than in infants, and to become chronic. This would account for the higher mean multiplicity of infection of 5 in asymptomatic children aged 2–7 years from the same village (BECK *et al.*, 1997). There is also evidence that high multiplicity is protective (AL-YAMAN *et al.*, 1997; BECK *et al.*, 1997). In older children from the same study site, multiplicity was associated with a reduced risk of clinical malaria, suggesting that existing infections cross-protect against superinfecting parasite clones. Such protection, termed concomitant immunity or premunition (SERGENT & PARROT, 1935), may be conferred by persistent, low-level parasitaemia. The absence of premunition in infants may in turn be a major factor contributing to their greater vulnerability to clinical malaria (TANNER *et al.*, 1999).

In a parallel study to this, we genotyped blood samples from children aged 2–7 years from Idete, which had

Table 4. Mean parasite density of *Plasmodium falciparum* according to multiplicity of infection[a]

Multiplicity	All genotypes No.	All genotypes Density[c]	3D7-like[b] genotypes No.	3D7-like[b] genotypes Density[c]	FC27-like[b] genotypes No.	FC27-like[b] genotypes Density[c]
0	–	–	30	461 (184–1151)	38	916 (403–2083)
1	46	687 (323–1461)	41	1525 (676–3440)	48	1284 (612–2694)
2	32	1742 (708–4288)	30	2055 (885–4769)	17	5825 (2237–15169)
3	17	955 (274–3331)	7	9471 (1832–48952)	6	511 (20–12758)
4	6	8822 (3741–20804)	1	25009	–	–
5	7	14228 (5063–39989)	–	–	–	–
6	1	25009	–	–	–	–

[a]There was no statistically significant interaction between age and multiplicity in their effect on parasite density ($F_{1,101}$=0·11, P=0·7).
[b]Densities were calculated for samples which included the respective allelic family irrespective of the presence of alleles belonging to the other allelic family.
[c]Geometric mean parasitaemia per μL (95% confidence interval in parentheses).

been collected in a cross-sectional survey to form part of the placebo group in the SPf66 vaccination trial (BECK *et al.*, 1997). Both data sets are comparable, since genotyped samples were derived from the same village and the same time period, except that older children were treated with pyrimethamine/sulfadoxine. Despite the treatment, which allows less time for accumulation of new infections, the cohort of older children had a much higher mean multiplicity. To reconcile the results from both age groups, it has to be assumed that multiplicity of infection increases gradually from the age of one year onwards. The non-significant increase observed at the end of the first year of life could be intrepreted as a precursor of the increase in mean multiplicity, which then reaches a peak of 5 PCR-detected infections per carrier at the age of about 3 years (SMITH *et al.*, 1999a).

Malaria morbidity depends, among other contributing factors such as host immune status, on exposure to new, less common, or more virulent *P. falciparum* variants (GUPTA *et al.*, 1994). Several studies have been undertaken to determine associations between genetic characteristics of parasites and morbidity (ENGELBRECHT *et al.*, 1995; CONTAMIN *et al.*, 1996; AL-YAMAN *et al.*, 1997). A study in patients with severe malaria from Senegal, in which 7 polymorphic marker genes were investigated, revealed that all isolates were genetically distinct; thus no single virulent 'strain' was detected (ROBERT *et al.*, 1996). In the present study, extensive genetic diversity, with 50 difference *msp2* genotypes, was found in all 260 community samples and fever cases. Frequencies of parasite genotypes from asymptomatic infants were compared to those from febrile infants in order to detect preferential carriage of any genotype. Although the K1 genotype appeared twice as often in febrile cases as in asymptomatic infants, the statistical analysis which allowed for the large numbers of comparisons made suggested that the apparent differences in virulence between genotypes were chance fluctuations.

The extensive diversity of *msp2* reduces the statistical power of a study to identify single virulent genotypes. Such studies have more power to analyse morbidity associations when individual alleles are grouped according to their allelic family. ENGELBRECHT *et al.* (1995) found that, in children from Papua New Guinea (PNG) FC27-like genotypes were twice as likely to be found in symptomatic malaria cases as were alleles of the 3D7 family. This corresponds to the results from a separate prospective study in PNG including individuals under 18 years, in which protection from clinical malaria was associated with the presence of 3D7-type parasites (AL-YAMAN *et al.*, 1997). In the study of older children from Idete (BECK *et al.*, 1997), parasite densities were found to be significantly higher in infections comprising FC27-like alleles only, and these were found predominantly in cases (12 of 14 cases) (unpublished data). In contrast, the present study showed no statistically significant difference between allelic families with respect to relationships with clinical malaria or parasite densities.

A series of field studies, including this one, has now confirmed the epidemiological significance of multiplicity of infection (AL-YAMAN *et al.*, 1997; BECK *et al.*, 1997; FRASER-HURT *et al.*, 1999). The comparison of multiplicity in different age groups suggests that the change in immunological status from that of the infant to that of a semi-protected child occurs, in an area of high transmission, between one and 2 years of age. The development of premunition, indicated by an increasing number of multiple infections, highlights this transition. This has implications for control measures against malaria, which must aim to protect very young children from infections which cause disease, but without compromising the subsequent acquisition of multiple infections and, thus, premunition.

Acknowledgements

We thank the population of Idete for their co-operation, which made this study possible, and Mr Charles Leuteli and his team from the Idete dispensary. We are grateful to the staff at the Ifakara Health Research and Development Centre for their efficient and skilful collaboration during data collection, and Professor W. L. Kilama, Director General of the National Institute for Medical Research (NIMR), Tanzania, for his support of this study. Research clearance, including ethical clearance, was obtained from the Medical Research Co-ordination Committee of NIMR through the Tanzanian Commission for Science and Technology (COSTECH) (NSR/RCA90). The investigation received financial support from the participating institutions, the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), the Friedrich Miescher Institute, Basel, Switzerland, the Jubilee Foundation of the Swiss Tropical Institute, and the Swiss Agency for Development and Co-operation. Dr Kitua was a TDR research training fellow. Dr Felger was supported by Swiss National Foundation grant 3200-045616.

References

Achidi, E. A., Salimonu, L. S., Asuzu, M. C., Berzins, K. & Walliker, O. (1996). Studies on *Plasmodium falciparum* parasitemia and development of anemia in Nigerian infants during their first year of life. *American Journal of Tropical Medicine and Hygiene*, **55**, 138–143.

Al-Yaman, F., Genton, B., Reeder, J. C., Anders, R. F., Smith, T. & Alpers, M. P. (1997). Reduced risk of clinical malaria in children infected with multiple clones of *Plasmodium falciparum* in a highly endemic area: a prospective community study. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **91**, 602–605.

Beck, H.-P., Felger, I., Huber, W., Steiger, S., Smith, T., Weiss, N., Alonso, P. L. & Tanner, M. (1997). Analysis of multiple *Plasmodium falciparum* infections in Tanzanian children during the trial of the malaria vaccine SPf66. *Journal of Infectious Diseases*, **175**, 921–926.

Contamin, H., Fandeur, T., Bonnefoy, S., Skouri, F., Ntoumi, F. & Mercereau-Puijalon, O. (1995). PCR typing of field isolates of *Plasmodium falciparum*. *Journal of Clinical Microbiology*, **33**, 944–951.

Contamin, H., Fandeur, T., Rogier, C., Bonnefoy, S., Konaté, L., Trape, J.-F. & Mercereau-Puijalon, O. (1996). Different genetic characteristics of *Plasmodium falciparum* isolates collected during successive clinical episodes in Senegalese children. *American Journal of Tropical Medicine and Hygiene*, **54**, 623–643.

Cox, M. J., Kum, D. E., Tavul, L., Narara, A., Raiko, A., Baisor, M., Alpers, M. P., Medley, G. F. & Day, K. P. (1994). Dynamics of malaria parasitaemia associated with febrile illness in children from a rural area of Madang, Papua New Guinea. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **88**, 191–197.

Desowitz, R. S., Elm, J. & Alpers, M. P. (1993). *Plasmodium falciparum*-specific immunoglobulin G (IgG), IgM, and IgE antibodies in paired maternal–cord sera from east Sepik Province, Papua New Guinea. *Infection and Immunity*, **61**, 988–993.

Engelbrecht, F., Felger, I., Genton, B., Alpers, M. & Beck, H.-P. (1995). *Plasmodium falciparum*: malaria morbidity is associated with specific merozoite surface antigen 2 genotypes. *Experimental Parasitology*, **81**, 90–96.

Felger, I., Tavul, L. & Beck, H.-P. (1993). *Plasmodium falciparum*: a rapid technique for genotyping the merozoite surface protein 2. *Experimental Parasitology*, **77**, 372–375.

Felger, I., Tavul, L., Kabintik, S., Marshall, V., Genton, B., Alpers, M. & Beck, H.-P. (1994). *Plasmodium falciparum*: extensive polymorphism in merozoite surface antigen 2 alleles in an area with endemic malaria in Papua New Guinea. *Experimental Parasitology*, **79**, 106–116.

Felger, I., Irion, A., Steiger, S. & Beck, H.-P. (1999). The epidemiology of multiple *Plasmodium falciparum* infections 2. Genotypes of merozoite surface protein 2 of *Plasmodium falciparum* in Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **93**, supplement 1, S1/3–S1/9.

Fraser-Hurt, N., Felger, I., Edoh, D., Steiger, S., Mashaka, M., Masanja, H., Smith, T., Mbena, F. & Beck, H.-P. (1999). The epidemiology of multiple *Plasmodium falciparum* infections 9. Effect of insecticide-treated bed nets on haemoglobin values, prevalence and multiplicity of infection with *Plasmodium falciparum* in a randomized controlled trial in Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **93**, supplement 1, S1/47–S1/51.

Gupta, S., Hill, A. V., Kwiatkowski, D., Greenwood, A. M.,

Greenwood, B. M. & Day, K. P. (1994). Parasite virulence and disease patterns in *Plasmodium falciparum* malaria. *Proceedings of the National Academy of Sciences of the USA*, **91**, 3715–3719.

Hill, W. G. & Babiker, H. A. (1995). Estimation of numbers of malaria clones in blood samples. *Proceedings of the Royal Society of London, Series B, Biological Sciences*, **262**, 249–257.

Kitua, A. Y., Smith, T., Alonso, P. L., Masanja, H., Menendez, C., Urassa, H., Kimario, J. & Tanner, M. (1996). *Plasmodium falciparum* malaria in the first year of life in an area of intense and perennial transmission. *Tropical Medicine and International Health*, **1**, 475–484.

Kwiatkowski, D. (1991). Cytokines and anti-disease immunity to malaria. *Research in Immunology*, **142**, 707–712.

Ntoumi, F., Contamin, H., Rogier, C., Bonnefoy, S., Trape, J. F. & Mercereau-Puijalon, O. (1995). Age-dependent carriage of multiple *Plasmodium falciparum* merozoite surface antigen-2 alleles in asymptomatic malaria infections. *American Journal of Tropical Medicine and Hygiene*, **52**, 81–88.

Pasvol, G., Weatherall, D. J. & Wilson, R. J. M. (1977). Effects of foetal haemoglobin on susceptibility of red cells to *Plasmodium falciparum*. *Nature*, **270**, 171–173.

Rasheed, F. N., Bulmer, J. M., De Francisco, A., Jawala, M. F., Jakobsen, P. H., Jepson, A. & Greenwood, B. M. (1995). Relationship between maternal malaria and malarial immune responses in mothers and neonates. *Parasite Immunology*, **17**, 1–10.

Robert, F., Ntoumi, F., Angel, G., Candito, D., Rogeri, C., Fandeur, T., Sarthou, J.-L. & Mercereau-Puijalon, O. (1996). Extensive genetic diversity of *Plasmodium falciparum* isolates collected from patients with severe malaria in Dakar, Senegal. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **90**, 704–711.

Rogier, C., Commenges, D. & Trape, J. F. (1996). Evidence for an age-dependent pyrogenic threshold of *Plasmodium falciparum* parasitemia in highly endemic populations. *American Journal of Tropical Medicine and Hygiene*, **54**, 613–619.

Sergent, E. & Parrot, L. (1935). L'immunité, la prémunition et la résistance innée. *Archives de l'Institut Pasteur d'Algérie*, **13**, 279–319.

Smith, T., Charlwood, J. D., Kihonda, J., Mwankusye, S., Billingsley, P., Meuwissen, J., Lyimo, E., Takken, W., Teuscher, T. & Tanner, M. (1993). Absence of seasonal variation in malaria parasitaemia in an area of intense seasonal transmission. *Acta Tropica*, **54**, 55–72.

Smith, T., Beck, H.-P., Kitua, A., Mwankusye, S., Felger, I., Fraser-Hurt, N., Irion, A., Alonso, P., Teuscher, T. & Tanner, M. (1999a). The epidemiology of multiple *Plasmodium falciparum* infections. 4. Age dependence of the multiplicity of *Plasmodium falciparum* infections and other malariological indices in an area of high endemicity. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **93**, supplement 1, S1/15–S1/20.

Smith, T., Felger, I., Kitua, A., Tanner, M. & Beck, H.-P. (1999b). The epidemiology of multiple *Plasmodium falciparum* infections 7. Dynamics of multiple *Plasmodium falciparum* infections in infants in a highly endemic area of Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **93**, supplement 1, S1/35–S1/39.

Smythe, J. A., Peterson, M. G., Coppel, R. L. Saul, A. J., Kemp, D. J. & Anders, R. F. (1990). Structural diversity in the 45 kilodalton merozoite surface antigen of *Plasmodium falciparum*. *Molecular and Biochemical Parasitology*, **39**, 227–234.

Tanner, M., Beck, H.-P., Felger, I. & Smith, T. (1999). The epidemiology of multiple *Plasmodium falciparum* infections. 1. General introduction. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **93**, supplement 1, S1/1–S1/2.