

Reduced risk of clinical malaria in children infected with multiple clones of *Plasmodium falciparum* in a highly endemic area: a prospective community study

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Abstract

A prospective community study in a highly malaria endemic area of Papua New Guinea found that infection with multiple *Plasmodium falciparum* genotypes was an indicator of lowered risk of subsequent clinical attack. The results suggest that concurrent or very recent infections provide protection from superinfecting parasites. The finding of an association between reduced risk of clinical malaria and infection with parasites of merozoite surface protein 1 (MSP-1) type RO33 or MSP-2 type 3D7 further suggests that the concomitant immunity is, at least in part, a consequence of a response to these major merozoite surface proteins.

Keywords: malaria, *Plasmodium falciparum*, genotypes, merozoite surface proteins, protection, superinfection, Papua New Guinea

Introduction

Acute attacks of *Plasmodium falciparum* malaria in partially immune individuals living in areas where malaria is highly endemic are probably associated with the acquisition of new infections (LINES & ARMSTRONG, 1992; MERCEREAU-PUJALON, 1996), possibly of novel variant antigenic type (GUPTA *et al.*, 1994). However, many *P. falciparum* infections fail to produce acute symptoms, even in age groups most vulnerable to clinical attacks (GREENWOOD *et al.*, 1987).

Recent studies have found differences between the parasitological picture in children with asymptomatic *P. falciparum* infections and that in clinical malaria patients from the same highly endemic areas (ENGELBRECHT *et al.*, 1995; CONTAMIN *et al.*, 1996; BECK *et al.*, 1997). Surprisingly, instead of a raised frequency of multiple infections during clinical episodes, these studies have all found the converse. The multiplicity of infection is higher in asymptomatic individuals. For example, in one of these studies (ENGELBRECHT *et al.*, 1995) it was found that the relative risk of clinical malaria varied depending upon whether the merozoite surface protein 2 (MSP-2) antigens of the infecting parasites belonged to the FC27 or 3D7 allelic families.

The reduced frequency of multiple infections in clinical cases has been considered to indicate a protective effect associated with antigenic cross-reaction between new inocula and the existing population of parasites circulating within the individual (BECK *et al.*, 1997). However, the assignment of causality in comparative studies with concurrent controls is problematic and it has not been possible to exclude the possibility that reduced multiplicity of infection is a consequence of, rather than a risk factor for, clinical malaria. Stronger evidence for causality in epidemiological studies is provided when the temporal order of events is recorded (BRADFORD-HILL, 1965). It is therefore useful to know whether multiple infections in asymptomatic individuals in a community survey are followed by periods of reduced risk of clinical attacks.

We report here a prospective study of the risk of clinical malaria in relation to the multiplicity of infection in children living in the same highly endemic area of Papua New Guinea as that studied by ENGELBRECHT *et al.* (1995). Morbidity surveillance was conducted by weekly household visits, thus further complementing the re-

sults of the previous studies, which considered only morbidity recorded at health facilities.

Materials and Methods

Sampling

A cohort of 236 children aged <18 years were recruited as described by AL-YAMAN *et al.* (1995). The children were invited to attend 3 cross-sectional surveys in October 1992, March 1993, and July 1993. At each survey, venous blood was collected into ethylenediamine-tetraacetic acid tubes for parasite typing.

Morbidity surveillance

Surveillance for clinical malaria was carried out via community-based case detection through weekly visits to the children enrolled in the study as described previously (AL-YAMAN *et al.*, 1995). Fever was defined as an axillary temperature $\geq 37.5^{\circ}\text{C}$, or a history of fever in the last 3 d. Blood films were prepared from all febrile children. Episodes which were detected only by surveillance at health facilities were not included in the present analyses.

For each child all weekly visits up to and including the time of the first clinical episode constituted the total visits at risk. Each visit was classified according to the parasitological status at the most recent cross-sectional survey. Any one child might contribute time at risk subsequent to any of the 3 surveys, but we considered only the first clinical episode satisfying the case definition for each child. Two distinct case definitions were used to define clinical *P. falciparum* malaria: (i) an episode of fever associated with a blood slide showing asexual stages of *P. falciparum*, and (ii) an episode of fever associated with a *P. falciparum* parasitaemia $>5000/\mu\text{L}$.

Parasitological methods

Thick and thin blood films prepared in the field were air-dried, stained with 4% Giemsa's stain, and examined for malaria parasites; 100 microscopical thick film fields were searched before a slide was considered negative. All 135 samples from cases in which asexual *P. falciparum* parasites were detected by microscopy were included in the study. These samples corresponded to 78% of the microscopically positive slides. We assumed that the parasitological pattern in other children with positive blood slides from whom samples were not available was similar to that in these samples. In order to obtain unbiased estimates of prevalence and of relative risks, we therefore chose at random 356 (78%) of the microscopically negative slides for inclusion in data analyses. Blood samples with negative slides were not genotyped.

Polymerase chain reaction typing

When asexual stages of *P. falciparum* were detected in blood films, a 10 μ L aliquot of the corresponding blood sample was lysed with 90 μ L of 8 M guanidine hydrochloride/0.1 M sodium acetate (pH 4.8), deoxyribonucleic acid (DNA) was extracted using Magic Miniprep™ columns (Promega) and an MSP-1/MSP-2 multiplex polymerase chain reaction (PCR) was performed, as described previously (REEDER & MARSHALL, 1994). This was followed by a second round of amplification with nested, individual MSP-1 and MSP-2 PCRs to increase sensitivity (AL-YAMAN *et al.*, in press). The second round MSP-1 and MSP-2 reactions of each sample were pooled, precipitated with ethanol and subjected to electrophoresis on 20 cm, 1.2% agarose gels and visualized by transillumination with ultraviolet light. Fragment sizes were then calculated by comparison with appropriate standards and controls. Size differences in MSP-1 and MSP-2 fragments were used to detect the presence of mixed infections in individual samples.

After visualizing the fragments, the gels were blotted on to nylon membranes (Hybond-N™, Amersham UK) which were probed sequentially with radiolabelled MSP-1 and MSP-2 family-specific probes by standard methods (FOLEY *et al.*, 1992; AL-YAMAN *et al.*, 1997) to determine allelic family type. The MSP-1 probes used were MAD20/FC27, K1 Wellcome and RO33, as described by SNEWIN *et al.* (1991). The MSP-2 probes used were FC27 and 3D7, as described by MARSHALL *et al.* (1994).

Results

Parasite DNA was amplified from 135 samples obtained during the cross-sectional surveys, corresponding to 78% of the parasitaemic blood films examined. The frequencies of single and multiple bands are given in the Table. Multiple bands were more frequently observed when MSP-1 rather than MSP-2 was amplified. In only 3 samples were single infections detected using MSP-1 but multiple infections using MSP-2; 19 samples produced multiple bands using MSP-1 but only single bands using MSP-2. It appeared, therefore, that under these conditions the MSP-1 PCR was a more sensitive indicator of the presence of multiple clones than the MSP-2 reaction.

The prevalences of single and multiple infections identified using MSP-1 amplification are shown according to age in the Figure, A. As in other studies in the same area (SMITH *et al.*, 1994; GENTON *et al.*, 1995a), parasite prevalence in children increased over most of the age range. The age trend with single infections was similar to that with multiple infections. Similarly, the prevalences of individual allelic families of both MSP-1 and MSP-2 showed age dependences similar to that of the total infections (Figure).

The incidence of morbidity showed a strong age dependence similar to that found in previous studies in the same area (SMITH *et al.*, 1994; GENTON *et al.*, 1995b). Therefore analyses of the risk of clinical malaria in relation to parasitological status (Table) were adjusted for age effects.

Similar relationships were found between parasitological status at the cross-sectional surveys and subsequent malaria morbidity risk, irrespective of which of the 2 definitions of clinical malaria was used (Table). Overall, fever episodes with patent *P. falciparum* parasites were detected at 0.57% of the household visits, and fever with *P. falciparum* densities greater than 5000/ μ L at 0.32% of visits. The presence of patent *P. falciparum* infection in general, MSP-2 type FC27 and MSP-1 types MAD20 or K1 infections in particular, did not affect the risk of subsequent clinical episodes. However, MSP-2 type 3D7 and MSP-1 type RO33 parasites were associated with a reduction in the risk of subsequent episodes. The effect with 3D7 was, however, statistically significant only when the more specific case definition for clinical malaria was used (fever with >5000 parasites/ μ L).

When only a single infecting clone was detected by PCR, the prospective risk of clinical malaria was higher than that in children who were not initially parasitaemic by microscopy (Table). However, the risk in children with multiple infections was much lower than that in either aparasitaemic children or in those with single infections, irrespective of whether multiplicity was assessed using MSP-1 or MSP-2 typing.

A total of 33 PCR-positive individuals could be followed at the next cross-sectional survey and parasites amplified from their further samples. Of 14 with single clone infections at the initial survey, 7 had multiple clones detected at the follow-up survey. Four of 19 individuals who initially had multiple clones detected had

Table. Recorded malaria morbidity rates by parasitological status at cross-sectional surveys

Parasitological status	Fever and any patent <i>P. falciparum</i> parasitaemia				Fever and <i>P. falciparum</i> parasites >5000/ μ L				
	No. of baseline slides ^a	Visits at risk ^b	Episode/Episodes	100 visits	<i>P</i> ^c	Visits at risk ^b	Episodes	100 visits	<i>P</i> ^c
Analyses (including follow-up of children with negative slides)									
Overall	491	9800	56	0.57	—	10044	37	0.37	—
Any parasite	135	2735	15	0.55	0.98	2820	9	0.32	0.7
FC27 (MSP-2)	92	1828	11	0.60	0.9	1873	8	0.43	0.7
3D7 (MSP-2)	90	1940	6	0.31	0.11	1989	2	0.10	0.017
MAD20 (MSP-1)	77	1593	9	0.56	0.9	1638	6	0.37	0.9
K1 (MSP-1)	90	1895	10	0.53	0.9	1963	4	0.20	0.2
RO33 (MSP-1)	26	524	0	0.00	0.01	524	0	0.00	0.04
Analyses (including follow-up of children with PCR positive slides)									
Single (MSP-1)	66	1224	10	0.82	—	1272	7	0.55	—
Multiple (MSP-1)	69	1511	5	0.33	0.042	1548	2	0.13	0.027
Single (MSP-2)	82	1555	13	0.84	—	1631	8	0.49	—
Multiple (MSP-2)	53	1180	2	0.17	0.009	1189	1	0.08	0.033

^aIn this study 3 cross-sectional baseline surveys were carried out. Thus each child could be observed up to 3 times (when a slide was taken). Corresponding to each of these baseline observations was a follow-up period which lasted either until a malaria episode occurred or until the next survey. The numbers in the Table are the numbers of slides from all of the baseline surveys which were followed-up.

^bThe number of weekly visits up to and including the visit at which the first clinical episode was detected.

^cLikelihood χ^2 ratio test, one degree of freedom in an age-adjusted Poisson regression model testing whether the prospective risk of morbidity differed between (i) children with the infection specified and those without that infection in the section including children with negative slides, and (ii) single and multiple infections in the section including children with PCR (polymerase chain reaction) positive slides.

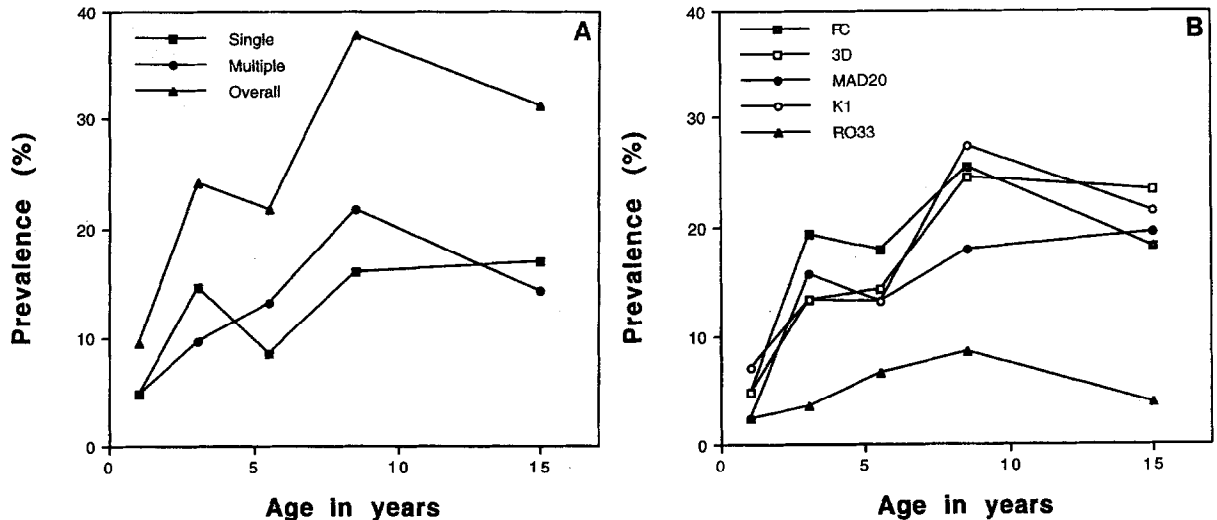


Figure. Prevalence by age of (A) single and multiple clone infections of *Plasmodium falciparum* and (B) MSP-1 (MAD20, K1, RO33) and MSP-2 (FC, 3D) allelic family types.

only single clones detected at the follow-up; multiple clones were again detected in the other 15 children.

Discussion

Previous studies of the genetic diversity of malaria parasites in the host as a risk factor for clinical malaria have compared malaria patients with control individuals from the same community. The design of these studies was appropriate for the identification of virulent genotypes but it was not optimal for the identification of protective factors. The finding that multiple infections were less frequent in the clinical cases was unexpected, and the hypothesis that a high multiplicity of infections protect against clinical attacks caused by superinfections could not be proven with this study design. It was not clear whether low multiplicity of genotypes, as identified by PCR amplification, was a cause or a consequence of clinical malaria.

Malaria patients seeking treatment are likely to be a selected group favourably disposed towards the health services, and are likely to have been treated more frequently in the past than other members of the community. This could explain why they are less likely to be infected with multiple clones of parasites. In the present study we have excluded this potential bias by considering only clinical malaria cases detected by household visits.

Clinical malaria in the partially immune child occurs when high parasite densities are reached. If, as seems likely, the expansion of a single parasite clone is responsible for the symptoms, this clone will provide most of the template for the PCR reaction, making it difficult to detect low density co-infections. There is also evidence that fever itself has an anti-parasitic effect (KWIATKOWSKI, 1989), and that clinical malaria triggers a complex cascade of cellular and humoral responses which also have marked anti-parasitic effects (KWIATKOWSKI, 1991). It follows that the pathology of malaria may result in a comparable reduction in densities of all clones of the parasite, which might well eliminate low-density clones. In the study by BECK *et al.* (1997), these alternatives to the hypothesis of a protective effect of multiple infections were dismissed because they could not readily explain the absence of the effect in children vaccinated with the SPf66 malaria vaccine.

The results of the present study support the interpretation that multiple infections are protective. The lower incidence of clinical attacks during follow-up of children with multiple infections clearly indicated that existing infections provided clinical protection against superin-

fecting parasites. This is not contradicted by the lower risk in children with no patent infection compared to those with single infecting clones: heterogeneities in exposure to infective bites within the study area are likely to mean that a subset of the children were at relatively low risk of both infection and clinical attacks because of low exposure.

The rate of malaria episodes for individuals with negative blood slides (0.58/100 weeks) lay between those for individuals with single and multiple infections. This was presumably because negative slides reflect lower exposure. If a subset of individuals were not exposed at all, they would obviously have negative initial slides and no subsequent malaria morbidity. On the other hand, individuals with negative initial slides, as a result of recent clearance of infection, but substantial exposure would be expected to have high subsequent morbidity. These 2 groups could not be distinguished in the present analyses.

Amplification of closely spaced longitudinal samples from the same individuals (DAUBERSIES *et al.*, 1996) has shown that, in areas with high rates of transmission, there was a rapid turnover of individual parasite populations. Although our data on the rates of change between single and multiple infections are limited, they do indicate that individual children do not remain fixed in one or the other of these categories. The fact that we could demonstrate a significant protective effect associated with multiple infections therefore suggests that it is concurrent or very recent infections which provide clinical protection, rather than an immunological response induced by a long history of high multiplicity infections.

The finding that the protective effect was specifically associated with parasites belonging to the 3D7 (MSP-2) allelic family corresponds to the previous results ENGELBRECHT *et al.* (1995), although in that study it was not possible to distinguish between a protective effect of 3D7 (MSP-2) parasites and enhanced virulence of FC27 (MSP-2) parasites. This finding, together with the apparent protective effect of RO33 (MSP-1) parasites, encourages the hypothesis that concomitant immunity is a consequence of immune responses against polymorphic epitopes of the major merozoite proteins themselves, rather than that these alleles simply act as markers for multiplicity of infections.

At the same time, this form of protection is clearly not the only mechanism of naturally acquired clinical immunity against *P. falciparum*. Even in areas of the highest endemicity, the build up of overall clinical immunity takes several years, whilst multiple infections can be ac-

quired rapidly even after effective antimalarial treatment (BECK *et al.*, 1997). The immunity associated with co-infecting parasites seems to be an important component of the overall battery of protective responses available to the partially immune host.

Acknowledgements

This project was supported by the US Agency for International Development, grant no. 9365967.89, the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, and the National Health and Medical Research Council of Australia. We thank the children and parents who participated in this study, the staff of Kunjingini Health Centre, the Papua New Guinea Institute of Medical Research field staff in Maprik, and the microscopists in Madang. We also thank Peter Beck for helpful comments on the manuscript. Data analysis was supported by the Swiss National Science Foundation.

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Received 5 December 1996; revised 6 February 1997; accepted for publication 6 February 1997