Chronic *Plasmodium falciparum* infections in an area of low intensity malaria transmission in the Sudan

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SUMMARY

Chronic *Plasmodium falciparum* malaria infections in a Sudanese village, in an area of seasonal and unstable malaria transmission, were monitored and genetically characterized to study the influence of persistent infection on the immunology and epidemiology of low endemicity malaria. During the October–December malaria season of 1996, 51 individuals out of a population of 420 had confirmed and treated *P. falciparum* malaria in the village of Daraweesh in eastern Sudan. In a cross-sectional survey carried out in December 1996, an additional 6 individuals were found to harbour a microscopically negative but polymerase chain reaction (PCR)-positive *P. falciparum* infection. On 1 January 1997, a cohort of 43 individuals aged from 9 to 53, recruited from this group of recently malaria-infected individuals agreed to donate fortnightly blood samples for the next 9 months, the first 6 of which constitute the long Sudanese dry season when transmission falls to undetectable levels. Each blood sample was tested for the presence of persistent malaria infection by microscopy and PCR. Parasite-positive samples were genotyped using PCR assays that detect allelic polymorphism at the MSP-1, MSP-2 and GLURP marker gene loci. Of 43 individuals 16 were found to maintain chronic *P. falciparum* infections which were continuously genetically characterized.

Key words: *Plasmodium falciparum*, chronic infections, unstable malaria, premunition, Sudan.

INTRODUCTION

For the last 10 years field studies have been carried out in Daraweesh village in eastern Sudan, aimed at understanding parasite population dynamics and human immune responses to malaria infections in an area of seasonal and unstable transmission. The dominant influence on malaria transmission in the Sahel and northern savannah belts of Sudan is the short summer rainy season, in which 80–90% of precipitation falls in July and August. The rains are followed by malaria outbreaks of varying degrees of severity, where the great majority of cases are due to *P. falciparum* and occur in October and November (Theander *et al*. 1990). Transmission drops around 8–10 weeks after the last heavy rains and new cases are rare after late November.

Studies in Daraweesh have demonstrated that a significant proportion of the population harbour asymptomatic infections detectable by transmission season-associated rises in anti-malaria antibody titres (El Hassan *et al*. 1995; Cavanagh *et al*. 1998; Giha *et al*. 1998). Parasitological confirmation of the presence of persistently microscopically negative but PCR-positive individuals has been obtained (Roper *et al*. 1996, 1998; Babiker *et al*. 1998). These results were unexpected since malaria in Daraweesh does not show the pattern of age stratification of clinical malaria incidence characteristic of areas of holoendemic transmission. Furthermore, individuals living in areas of unstable and low intensity transmission have not traditionally been considered to acquire significant immunity to the disease (MacDonald, 1957).

This study was designed to analyse persistent *P. falciparum* infections by genotyping a longitudinal series of samples donated fortnightly for 9–12 months by a closely monitored cohort of individuals in Daraweesh. Its objectives were to measure the duration of infection and fluctuations in parasitaemia within persistent infections and to ascertain whether such infections are in fact continuously asymptomatic. The survival of genetically different *P. falciparum* clones within initially multi-clonal infections was also monitored. The extent to which chronic *P. falciparum* infections in Daraweesh

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provide immune premunition (Sergent & Parrot, 1935) is also considered in the light of recent analyses of the malaria premunition and the epidemiology of multiple clone infections under much higher levels of transmission in Tanzania (Smith *et al*. 1999).

MATERIALS AND METHODS

Study population and epidemiology

Daraweesh village is in Gedaref State in eastern Sudan, 450 km from Khartoum and 16 km from Gedaref town. It is inhabited by approximately 420 Arabic speakers of Fulani ethnic origin whose ancestors settled in this area a century ago. The village economy is based on agriculture. Malaria transmission is markedly seasonal and unstable and annual peak parasite prevalence ranges from 1 to 40 % in different years. *P. falciparum* is responsible for > 96 % of malaria cases, the remainder being *P. vivax* and *P. malariae*. *Anopheles arabiensis* is the sole vector.

Clinical surveillance

Almost all malaria cases in Daraweesh are clinically uncomplicated, probably because of the continuous monitoring by a health team who visits the village on a daily basis and provides free drug treatment. The health team consists of a doctor, resident village health worker and a microscopist. Blood slides were made at the fortnightly sample collections and from individuals complaining of symptoms suggestive of malaria. Patients were considered to have clinical malaria if parasites were detected and either their body temperature measured > 37·5 °C or the patient complained of recent fever or other malaria symptoms. Microscopy was used to measure the number of the circulating parasites in individual infections throughout the study period. Parasites were counted per 300 leukocytes and standardized as parasites/μl (Trape, 1985; Petersen *et al*. 1996). Treatment was initiated immediately after diagnosis.

Study design

The 43 individuals in the cohort were recruited from people who either had a malaria attack during the transmission season or were PCR positive for *P. falciparum* malaria at the end of the malaria season in the first week of December 1996. Samples were taken at days 0, 3, 7 and 30 following diagnosis during the 1996 malaria season and regular fortnightly sampling was then instituted in the dry season and following rainy season between January and September 1997.

DNA extraction and PCR analysis

All positive infections were PCR genotyped and PCR fragments were also subcloned for DNA sequencing to estimate the number of parasite clones and the genetic stability of the parasite population within individual infections. Red blood cells were separated from plasma in the field and mixed with an equal volume of parasite freezing solution (28 % glycerol, 3 % sorbitol, 0·65 % NaCl), shipped in liquid nitrogen and then stored at −70 °C. Filter paper samples were kept in individual plastic bags at 4 °C. Parasite DNA for PCR from both venous blood samples and filter papers was extracted using the extraction protocols of Foley, Ranford-Cartwright & Babiker (1992) and Wooden *et al*. (1993). PCR reactions using ribosomal RNA gene primers to detect low level parasitaemias and using MSP-1, GLURP and MSP-2 primers to genotype infections followed the conditions described by Roper *et al*. (1998) and Cavanagh & McBride (1997).

Statistical analysis

χ^2 tests were used to test the significance of the different rates of fever measured in chronically infected and uninfected individuals in the cohort. Student's *t*-test was carried out to compare the significance of age distribution between the chronically infected and non-infected individuals. Linear regression analysis was carried out to correlate the effect of age, initial parasite density and sex difference on fever morbidity among the two groups.

RESULTS

Duration of infections

Fig. 1 summarizes the asexual parasitaemias observed in the 43 cohort members from their first appearance during the autumnal Sudanese malaria season. Six of these cohort members did not have a clinical malaria attack during the 1996 malaria season but were found by PCR to be infected at a subpatent level during a cross-sectional screen of all inhabitants in the first week of December 1996. For the purpose of graphical presentation of the data, PCR positive infections are defined as 1 parasite/l although the actual parasitaemia is not quantifiable by this method. Presenting parasitaemias in the clinically ill individuals ranged widely from 600 to 210000 parasites/l blood. The age of individuals carrying chronic infections is illustrated on the individual graphs in Fig. 1. A significant overall correlation was detected between age and the initial parasite density at malaria diagnosis, the younger age groups tending to present with higher average parasitaemias. However, analysing the age distributions by linear regression, no significant effect of age distribution on the likelihood of developing chronic infection was observed ($P = 0{\cdot}337$). Neither was significant correlation observed between sex and likelihood of developing chronic malaria ($P = 0{\cdot}164$).

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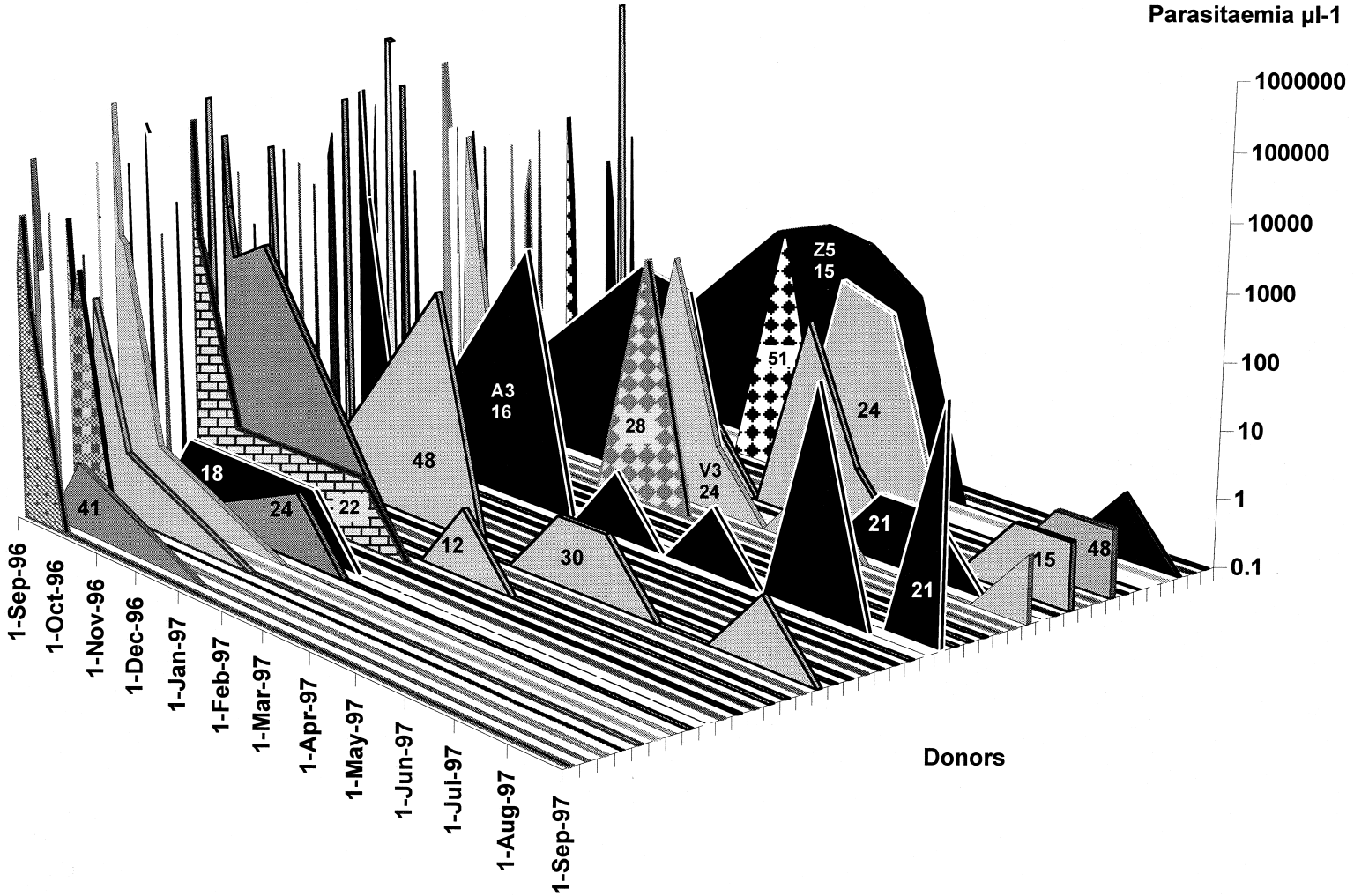

Fig. 1. Three-dimensional representation of the fluctuations in asexual parasitaemia over the 10 month monitoring period in the cohort of 43 Daraweesh villagers. The figure of 1 parasite/l is assigned to PCR positive/slide negative infections. Black shading represents infections treated with both chloroquine and pyremethamine/sulphadoxine. The assigned numbers represent the age of the chronically infected individuals.

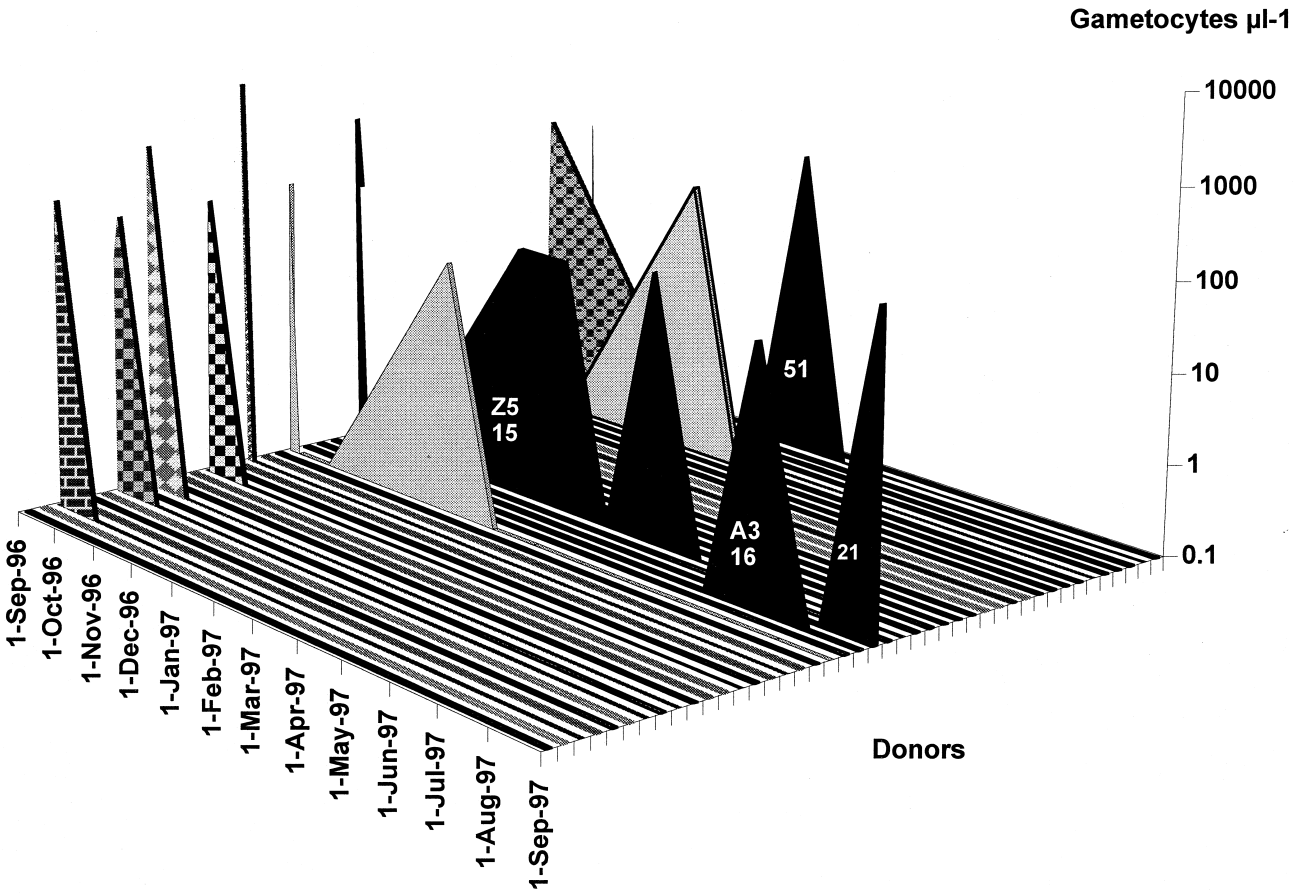

Fig. 2. Observed gametocytaemia in the 43 cohort members, at the indicated dates during and after clinical infection. The black bars represent infections treated with both chloroquine and sulphadoxine/pyremethamine.

Most individuals (20/37, 54%) cleared their parasites within 3 days after commencing a standard course of chloroquine. Those still positive 3 days after diagnosis were treated with pyremethamine/sulphadoxine and 29/37 (78%) individuals had no detectable parasites by day 7. The 22% of patients who were still positive on day 7 all had symptomatic remission and were not re-treated unless factors such as vomiting had interrupted the full course of treatment. By day 30 only 1 individual still had a slide-positive parasitaemia although 7 were PCR positive.

On 1 January regular fortnightly monitoring of the cohort was initiated. Blood smears from cohort members were examined in the field although all collected blood samples were assayed for infection by PCR at the end of the study. The graph in Fig. 1 illustrates that a substantial minority of the cohort (16/43, 37%) harboured varying levels of *P. falciparum* infection through the dry season and up to the next transmission season in September 1997. Parasitaemias in these individuals fluctuated above and below the threshold of microscopic detection (around 50 parasites/l in our field laboratory) and of PCR detection. These infections were capable of long-term production of gametocytes. The graph in Fig. 2 illustrates microscopical detection and quantification of gametocytaemia in those members of the cohort whose blood films contained gametocytes at any point during the study period.

The rate of clearance of infection

Fig. 3 indicates the rate of clearance of *P. falciparum* malaria in 3 groups of patients. Graph A indicates the time taken for all individuals within the group of 43 Daraweesh cohort members to clear infections as represented by the proportion of the cohort with *P. falciparum* infection (detected by either blood film or PCR) in the months following the malaria season. The rapid decrease in the infected proportion between days 0 and 7 in Fig. 3A is due to drug treatment. To minimize the confounding effect of drug treatment on parasite clearance time, the total cohort was reduced to the group of 16 who maintained long-term infections after any effect of earlier drug treatment would be likely to have disappeared. Clearance times in this subcohort are presented in the graph B of Fig. 3. Parasite clearance in these two groups was compared with the proportion of patients remaining positive at succeeding intervals of time after a single infection with *P. falciparum* in a Puerto Rican study carried out by Earle *et al.* (1939) (Graph C). These data were based on monitored but untreated infections in children

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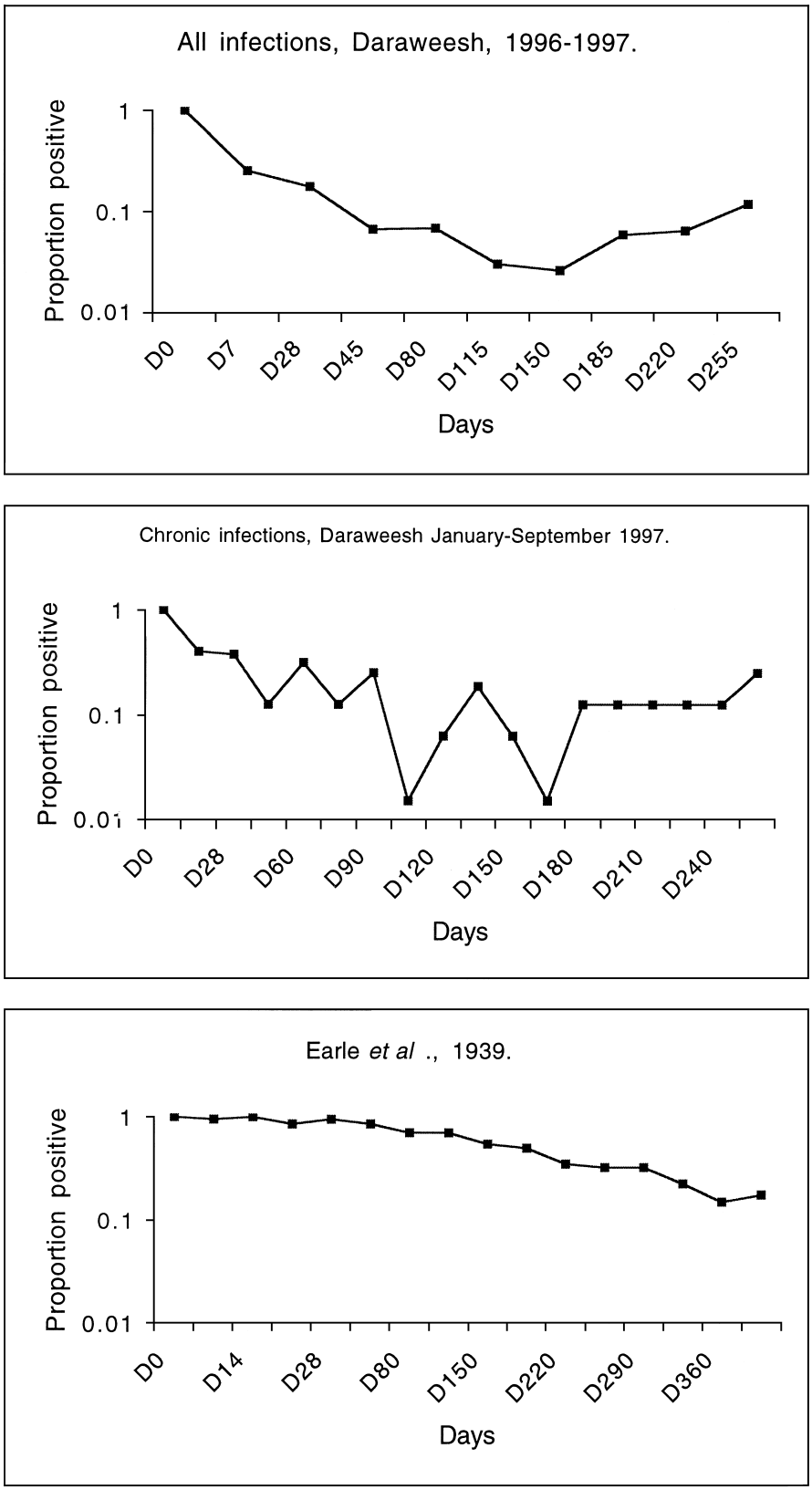

Fig. 3. Three graphs representing the proportions of individuals remaining infected with *Plasmodium falciparum* at succeeding intervals of time following single infections. (A) Representation of all data collected on 43 individuals in the Daraweesh cohort study during the transmission season of 1996 and the dry season of 1997. November 1 was taken to be the point when all cohort members were infected. (B) Data from the 16 individuals who remained chronically infected throughout the dry season. (C) Summary of the data on the observed period of infection in a group of *P. falciparum*-infected children taken from a Puerto Rican study carried out by Earle *et al*. (1939) and reproduced in Anderson & May (1991). No drug treatment was used in this study.

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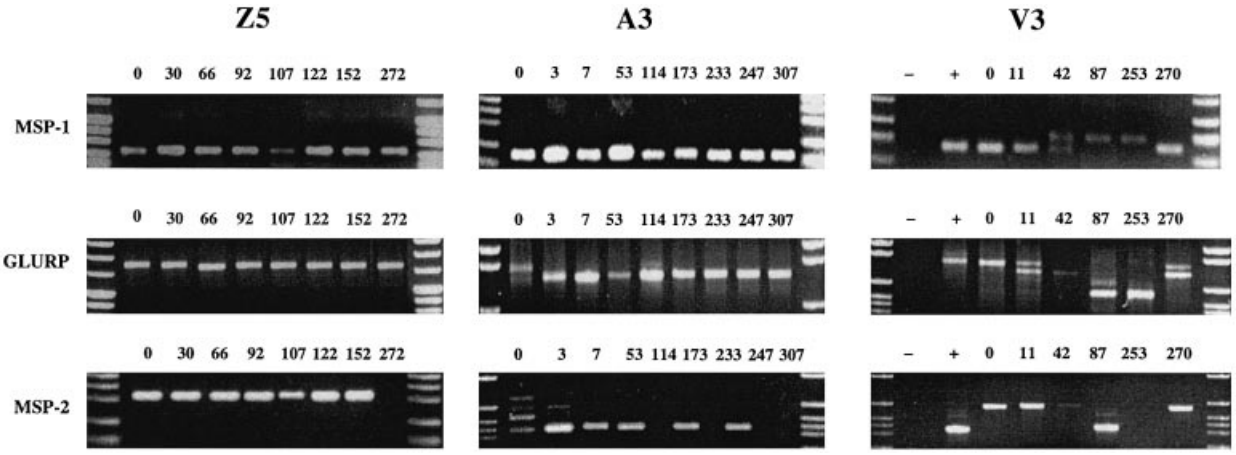

Fig. 4. Genotyping of isolates by PCR amplification of the *Plasmodium falciparum* MSP-1, MSP-2 and GLURP genes and separating amplified DNA products on 1·5 % agarose gels. The results shown were obtained from serial samples from 3 cohort members, (Z5, A3 & V3) collected between October 1996 and September 1997. 0, 3, 7 and 30 refer to the days following clinical infection during the transmission season. Subsequent numbers refer to samples taken during the dry season, numbered in days from the onset of the clinical episode.

from rural, malaria endemic areas admitted to a San Juan orphanage where there was no malaria transmission.

Clonal stability and turnover during chronic P. falciparum *infection*

The data presented in Figs 1–3 indicate that chronic infections of *P. falciparum* in Daraweesh, despite clinical treatment, last long enough to maintain a reservoir of infection in the 8–9 month period between eastern Sudanese rainy seasons. However, the above data do not prove that single infections actually persist throughout this period as they do not exclude the possibility of re-infection. (It is extremely difficult to detect feeding *An. arabiensis* females during the hot, dry season in Sudan (Omer & Cloudsley-Thompson, 1970; Hamad, unpublished observations).) To ascertain whether the infections detected during the follow-up sampling of the cohort remained the same or were different from the original clinical isolate, all infection samples were genotyped by PCR. Fig. 4 shows examples of PCR genotypes of the circulating parasites from three cohort members, sampled over 10 months.

P. falciparum infections can be classified as uniclonal, where only 1 allele at any of the 3-marker loci was detected at any sampling point during the course of the infection and multi-clonal, where at some point during the infection > 1 allele was detected at any one of the marker loci. The simplest pattern of chronic infection is that shown by cohort member Z5. This individual presented at day 0 with a uniclonal infection, as judged by the fact that only a single band was PCR amplified at each of the MSP-1, 2 and GLURP loci using primers hybridizing between regions known to have different lengths in different alleles. Sequence analysis of the MSP-1 product of all Z5 samples showed identical MAD 20 sequences at all points during the infection (data not shown). Despite chloroquine and pyremethamine/sulphadoxine treatment, parasite clearance and symptomatic recovery, Z5, remained chronically infected with the same, presumably drug resistant clone, for at least the next 272 days. Patient Z5's course of infection is labelled in Fig. 1. For the 120 days between the day 152 and day 272 sample, Z5 was apparently uninfected, although the reappearance of the same clone at day 272 indicates that the infection remained in a subpatent form. In all, 9/16 infections which became chronic in the cohort were continuously uni-clonal, with constant parasite genotypes from the point of first detected infection (X8, G4, VI, Z5, AE5, 2A4, 2P8, Z7, AA4). All of these infections were genetically different.

A different pattern of infection was noted in 4/16 individuals (A3, 2P11, Z8, 2O2). These individuals followed a course of infection similar to patient A3 (Figs 1, 4). Parasites in the sample donated when first reporting ill show a mixture of alleles, indicating that the individual was infected either by a mixture of genetically different sporozoites, or received super-infecting parasite inoculations. However, soon after commencing drug treatment (chloroquine and then pyremethamine/sulphadoxine), the complexity of the infection decreased and the chronic phase of an infection was maintained by a single clone. Patient A3's infection is represented by the saw-toothed parasitaemia course in Fig. 1. This infection lasted at least 307 days, although it was not continuously detectable in the (less sensitive) MSP-2 PCR assays (Fig. 4).

Three individuals (V3, 2H6 and C1) showed the most complex pattern of infection. In patient V3's infection at day 0, there was apparently a single product amplified at the MSP-1 and 2 loci. However, 1 major and 3 minor bands were amplified at the GLURP gene locus. At day 11 there was no change

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Table 1. Episodes of fever or fever complaints in uninfected and chronically infected individuals of the cohort in Daraweesh during the dry season of 1997

(The difference in age between the infected and uninfected groups was not significant. Among the chronically infected group, there was no significant difference in age between those who had or complained of fever and those who did not.)

	Measured fever or complaints of fever at follow-up visit	No measured fever or complaints of fever at follow-up visit	Total	Average age
Chronic infection	19 (average age 25·1)	203 (average age 26·3)	222	25·6
Cleared infection	23	327	350	23·1
Total	42	530	572	

at the MSP-1 and 2 loci but the relative intensity of the 4 GLURP bands had changed. By day 42, despite faint amplification of the GLURP and MSP-2 locus products, possibly due to an overall decrease in parasitaemia, a higher molecular weight band had appeared in the MSP-1 locus. At day 87 the predominant clone in the infection was different from that in the initial infection, with higher and lower molecular weight alleles at the MSP-1 and 2 loci respectively. Similar changes in the clonal composition of the infection were apparent in the day 253 and day 270 samples also. Whether these represented the appearance of clones which were not present in the original infection, or successive changes in the proportions of clones which were all present at day 0 is difficult to say, although the patterns visible in the initial GLURP genotypes may support the latter alternative.

Patient 2H6 showed the same type and size of the amplified fragments in all samples; however, sequence analysis of subclones made from the MSP-1 PCR product revealed 3 different MSP-1 Mad 20-type sequences (data not shown). Two related patients, B4 and B7, were first found to be infected with *P. vivax* although they were infected at different times. At day 3 of treatment both were also detected to be *P. falciparum* positive (with genetically different clones). B4 went on to become chronically infected with *P. falciparum* although his *P. vivax* infection was cleared.

Fever morbidity in chronic infections

To investigate whether chronic malaria infections increase the burden of malaria morbidity in this population or are to all intents truly asymptomatic, temperatures were measured at each of the bi-monthly follow up visits to all 43 cohort members. Individuals were also asked about their general health and specifically if they had experienced fever in the period since the last follow-up visit. The results of the fever enquiries in a total of 572 follow-up visits are summarized in Table 1. These figures indicate that the 16 individuals subsequently discovered to have been chronically infected did not have a significantly higher risk of experiencing fevers during the dry season than those who had completely cleared their infection ($P = 0{\cdot}375$). A linear regression analysis was carried out to test the effect of sex, or of increasing age on the likelihood of complaining of fever. No significant correlation with increasing age or with sex were obtained ($P = 0{\cdot}394$ and 0·364, respectively).

Chronic infections in Daraweesh and malaria premunition

Fig. 1 indicates that initial clinical infections are either completely cleared by treatment or develop into chronic infections with fluctuating parasitaemias not exceeding a few hundred parasites/l. Thus the immune response seems able to control 'secondary waves' of parasite replication at around 3–4 orders of magnitude lower parasite densities than is achieved in primary infections. A hypothesis invoking chronic multi-clonal infections to explain the contribution of antigenically diverse *P. falciparum* clones to conferring protective premunition against super-infection has recently been proposed (Smith *et al.* 1999). In Fig. 5 this hypothetical model is extended to compare Tanzanian infants and older children (living under entomological inoculation rates of 300–500 infective bites per year) with transient and chronic uni- and multi-clonal infections in Daraweesh where individuals receive around 1–2 infective bites per year.

Visual representation of this model requires that each of the frames constitutes an 'immunological space' representing the total immune response repertoire of one host and the circles correspond to the total antigenic repertoire of a single *P. falciparum* clone infecting that host. Size and shading intensity of the circles are proportional both to the length of infection with that clone and to the strength and persistence of host responses to the antigenic repertoire of that clone. The darker the shading, the stronger the response to these epitopes and the more protected the host is against super-infection by new infections bearing these epitopes.

The hypothesis predicts that very young children in high transmission areas such as parts of Tanzania (Frame A) are not as protected as older children (Frame B) from clinical malaria attacks (Smith *et al.*

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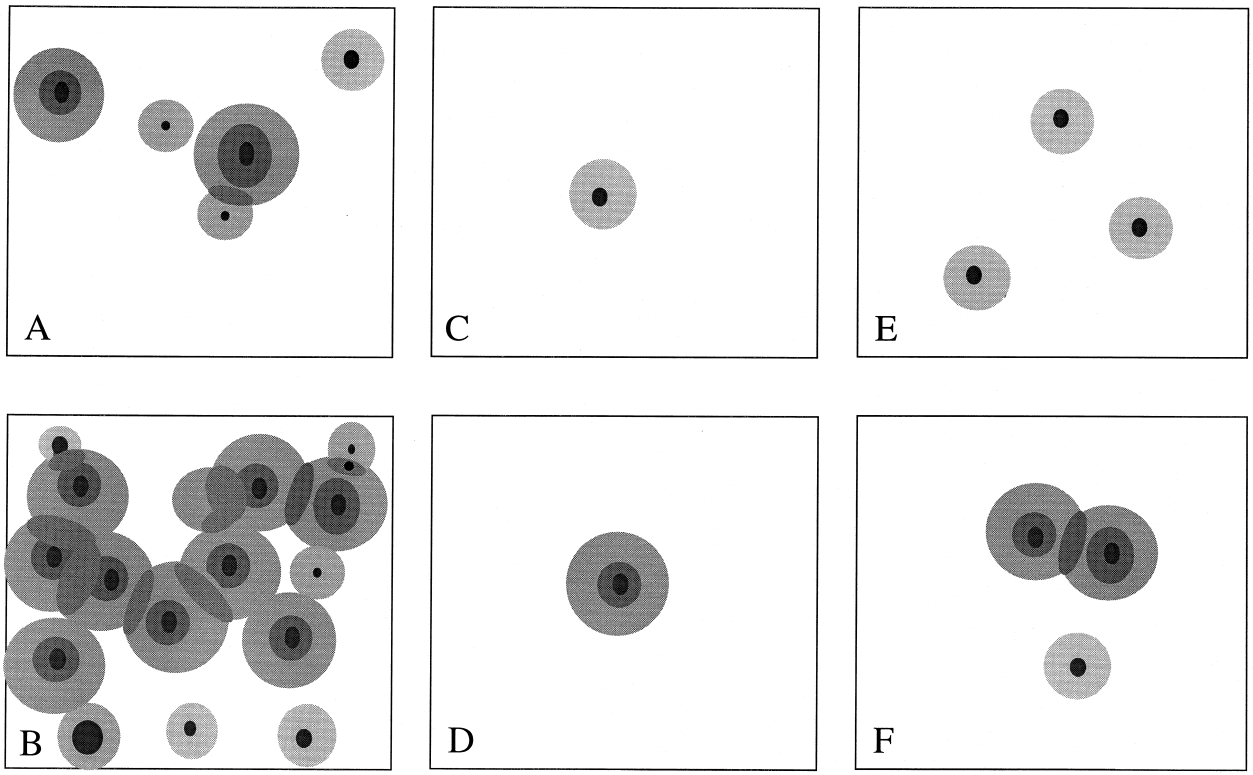

Fig. 5. Schematic representation of the multi-clonal induction of malaria premunition hypothesis of Smith, Felger, Tanner & Beck (Smith *et al.* 1999) used to compare varying states of clonal inoculation and immunity between an area of holo-endemic transmission (Tanzania) and Daraweesh.

1999). This is because both polyclonal and chronic malaria infections are required to generate the large number of epitope-specific responses necessary to block the establishment of new infections. However, neither drug-cured single clone or low-multiplicity infections in Daraweesh (Frames C & E), nor the chronic infections which may develop from such infections (Frames D & F) occupy enough 'immunological space' to confer significant protection against the very diverse range of antigenically distinct clones in the population.

DISCUSSION

The duration of infection and infectiousness in acute febrile protozoan infections is much longer than that observed in febrile viral diseases such as influenza and the common cold (Anderson & May, 1991). This study provides additional evidence that the duration of *P. falciparum* infection in the Sudanese Sahel is long enough to allow parasites inoculated during the transmission season to survive through the long, dry season. Such infections provide the reservoir for the next season's malaria outbreak (Roper *et al.* 1996; Babiker *et al.* 1998). The majority of chronic infections were observed to contain only 1 clone under these low-intensity transmission conditions and this seems no barrier to their long-term survival. The longest period of survival of a single clone that we have monitored was at least 272 days (patient Z5).

The data shown in Fig. 3 indicate that the average duration of infection was shorter in this study than that observed in a 1939 Puerto Rican study (Earle 1939). We attempted to minimize the effect of the drug treatment given in this study by examining the duration of infection in those found to be asymptomatically infected without having had a treated malaria attack. After an early decrease in the proportion of individuals remaining infected, the chronically infected proportion then stabilized before declining in a similar way to that observed in the Puerto Rican cohort. Since the treatment facilities available in Daraweesh are not representative, it can be assumed that the proportion of individuals remaining infected at succeeding intervals after single *P. falciparum* inoculations is significantly higher in other Sudanese communities.

Drug resistance is a factor in the establishment of chronic malaria infections. Six of 16 chronically infected individuals had chloroquine-resistant malaria and were treated with pyremethamine/sulphadoxine, compared to 8/27 chloroquine-resistant cases in those clearing their malaria completely. During the 1998 transmission season, of the total of 168 cases observed in the village, 38% were clinically chloroquine resistant and around 11% were also clinically pyremethamine/sulphadoxine resistant. It is possible that, in contrast to the situation 10–15 years ago (Bayoumi *et al.* 1989), both chloroquine and pyremethamine/sulphadoxine resistance have

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now crossed a threshold in many areas of Sudan whereby the reservoir of chronic infections available to seed each new seasonal outbreak is now predominantly composed of drug-resistant isolates.

Observations on the immune status of the population of Daraweesh are somewhat paradoxical. On the one hand, malaria case incidence is not heavily concentrated amongst young children and neither in 1997–98 nor in earlier studies has the occurrence of clinical malaria in an individual conferred any protection against malaria in the next transmission season (El Hassan *et al*. 1995; Roper *et al*. 1998; Giha *et al*. 1999). On the other hand, a significant proportion of the population show evidence of having had subclinical infections during the September–November period as detected by transmission season-associated rises in anti-malaria antibody titres (El Hassan *et al*. 1995; Cavanagh *et al*. 1998; Giha *et al*. 1998). Furthermore, age stratification in case incidence is not completely absent from Daraweesh as older adults and younger children suffer significantly fewer febrile malaria episodes than adolescents and young adults (Giha *et al*. 1999).

Our observations on low clone multiplicity/low transmission intensity in malaria in the Sudanese Sahel also differ from those described in field studies from higher transmission zones such as Papua New Guinea and Tanzania. In the Maprik, New Guinea study, the prospective risk of clinical malaria in children with multiple clone infections was much lower than in either aparasitaemic children or in those with single infections (Al-Yaman *et al*. 1997). A recent Tanzanian study also found this tendency for higher clone multiplicities to be associated with reduced frequency of clinical malaria. In addition, children with detectable *P. falciparum* infections when asymptomatic, were found to experience significantly fewer disease episodes than children who were parasite negative between episodes (Farnert *et al*. 1999). No correlation between age and number of circulating parasite genotypes was found in this study nor was there a trend for the number of genotypes to decrease in individuals over 4 years of observation.

A possible explanation of the clear lack of resistance to malaria in older Daraweesh children and adults may be offered by the multi-clonal induction of premunition hypothesis proposed by Smith and his colleagues (Smith *et al*. 1999). In this view, protection from clinical disease largely operates via a blockade against super-infection maintained by established chronic infections composed of numerous clones. Even chronic infections in Daraweesh would be predicted to be poorly protective because they are composed of one or a few clones that only induce immune responses against a relatively small number of variable parasite epitopes. Such infections might, however, protect against super-infection by parasites with antigenic similarities to those already experienced.

That immunity to high rates of super-infection requires both chronic and multi-clonal infection is an interesting hypothesis that redefines the concept of malaria premunition. Whether it is a complete interpretation of the complexities of parasite clone multiplicity and turnover in chronic infections in all age groups in endemic areas requires further field research. Alternative explanations of some PCR-based observations on clone multiplicity have been proposed (Arnot, 1998). The relative reduction in malaria incidence observed in adults in Daraweesh remains puzzling and difficult to completely explain by premunition against super-infection. Natural immunity to malaria is not understood. Models involving cumulative acquisition of long-term immunity to diverse variable antigens (Gupta & Day, 1994; Hviid, 1998), immunity to relatively conserved parasite antigens (Druilhe & Perignon, 1997) or mechanisms dependent on age and immunological maturity (Baird *et al*. 1991; Ntoumi *et al*. 1995) remain neither proven nor excluded.

This paper is dedicated to the late El Tayeb Hayati, our technician who died carrying out field work for this study. The generous co-operation of the people of Daraweesh, and the Malaria Administration of the Sudanese Health Ministry and the work of the Gedaref malaria team are gratefully acknowledged. We thank Margaret Mackinnon for statistical help and advice, David Cavanagh, Lisa Ranford-Cartwright, Mercy Sowa, George Clottey, Alison Creasey, Gwiria Satti, Hamza Babiker and Chris Ward for their valuable discussions and help. This work received funding from the UNDP/World Bank/WHO (TDR Project ID 960448) and the INCO-DC Programme of the European Union (IC18 CT97-0238). D. E. A. was also supported by a Senior Fellowship of the Wellcome Trust.

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