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New Approaches to the Serotypic Analysis of the Epidemiology of Plasmodium falciparum [and Discussion]

Karen P. Forsyth, R. F. Anders, D. J. Kemp, M. P. Alpers, G. A. T. Targett, D. J. Conway, R. M. May and D. A. J. Tyrrell

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New approaches to the serotypic analysis of the epidemiology of *Plasmodium falciparum*

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Considerable antigenic heterogeneity of *Plasmodium falciparum* has been demonstrated in natural parasite populations. However, very little is known about the relative virulence, transmission efficiency and prevalence over space and time of parasites expressing different serotypes of variant antigens. The recent application of recombinant DNA techniques to express a wide range of *P. falciparum* antigens in *Escherichia coli* has led to a better understanding of the molecular basis of antigenic diversity of a number of parasite proteins including the precursor to the major merozoite surface antigen (PMMSA) and the heat-stable S-antigens. Highly specific reagents such as DNA probes, monoclonal antibodies and polyclonal antisera to either cloned antigens or synthetic peptides have become available for serotypic analysis of natural parasite populations. With these reagents important epidemiological questions can now be asked concerning the population biology of different serotypes of *P. falciparum*. The use of the polymorphic S-antigen system as a serotypic marker to analyse the transmission dynamics of *P. falciparum* in Madang, Papua New Guinea (PNG) is discussed. Results of serotyping studies with the S-antigen system highlight the complexities of malaria transmission, which require consideration in the design of malaria vaccine trials.

1. Introduction

Plasmodium falciparum causes the most severe form of human malaria. Immunity develops slowly after repeated infection over many years, probably owing to the existence of many antigenically distinct forms of this parasite. Antigenic diversity of *P. falciparum* has been demonstrated by cross-immunity studies in chimpanzees with different geographic isolates of this parasite (Sadun *et al.* 1966) as well as by serological analyses of a number of antigens (Wilson *et al.* 1969; McBride *et al.* 1985; Marsh & Howard 1986). Recent studies of the molecular biology of *P. falciparum* have defined some of the genetic mechanisms whereby antigenic heterogeneity of malaria parasites is generated. At least three genetic mechanisms play a role. First, most genes of *P. falciparum* encode polypeptides that contain tandem repeats. Recombination events such as unequal crossing-over can lead to the spread of accumulated mutations from one repeat to another. For some genes, such processes have generated totally different repeat sequences in different isolates (Saint *et al.* 1987), although in other genes the repeats are conserved (Dame *et al.* 1984). As the repeats are the primary target of human antibodies, repeat variation is one way of generating antigenic diversity. The second mechanism is simple Mendelian segregation and recombination, which has been observed to occur between two genetically distinct clones of *P. falciparum* during the sexual phase of the malaria life cycle in the mosquito host (Walliker *et al.* 1987). Thirdly, generation of novel molecules by intragenic recombination during meiosis

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most likely accounts for the sequence diversity observed for the precursor to the major merozoite surface antigen (PMMSA) from different isolates of *P. falciparum* (Tanabe *et al.* 1987; Peterson *et al.* 1988).

Although we now have a better understanding of the molecular basis of antigenic diversity in *P. falciparum*, a number of questions remain unanswered concerning the population biology of parasites expressing different serotypes of variant antigens in endemic areas. Very little is known about the relative virulence, transmission efficiency and prevalence over space and time of antigenic variants of *P. falciparum*. Indeed, there is no functional definition of the term 'serotype' as applied to the description of the epidemiology of malaria. Molecular characterization of antigens of *P. falciparum* has shown that this parasite has a number of polymorphic antigens. The genes encoding some of these antigens lie on different chromosomes (Kemp *et al.* 1987) and will most likely undergo genetic recombination independently of each other. Therefore serotyping *P. falciparum* parasites by a single polymorphic antigen may only describe a sub-population of parasites that are heterogeneous with regard to other polymorphic antigens. The frequency of infections of mixed serotypes of polymorphic antigens in the human host and the rate at which genetic recombination occurs within natural parasite populations have yet to be determined.

The recent application of hybridoma technology and recombinant DNA techniques to characterization of malaria antigens has made available DNA probes, monoclonal antibodies and polyclonal antisera to either cloned antigens or synthetic peptides for serotypic and sequence analysis of polymorphic antigens of *P. falciparum* in natural parasite populations. For example, McBride *et al.* (1985) have used a panel of thirteen monoclonal antibodies to define seven serotypes of PMMSA, a candidate molecule for a malaria vaccine, in a large number of isolates of *P. falciparum* from around the world. DNA hydridization techniques utilizing oligonucleotide probes to variable sequences of dimorphic forms of PMMSA have also been used to study sequence variability of this antigen in a number of isolates of *P. falciparum* (Peterson *et al.* 1988). Diversity of the S-antigen system of *P. falciparum* has also been defined by specific antisera as well as by DNA hybridization and sequence data (Saint *et al.* 1987). The ready availability of serotyping reagents to PMMSA and S-antigens makes these two polymorphic antigens suitable markers for seroepidemiological investigations of t mission dynamics of sub-populations of *P. falciparum* parasites in endemic ar potential use of the S-antigen system for such studies is discussed in detail with data fr transmission experiments.

2. S-antigen system

The heat-stable S-antigens of *P. falciparum* exhibit extreme serological diversity (Wilson *et al.* 1969) as well as size and charge heterogeneity (Anders *et al.* 1983; Winchell *et al.* 1984; Howard *et al.* 1986). They are secreted into the parasitopherous vacuole of the mature schizont and are released into the circulation of infected individuals during schizogeny (Wilson *et al.* 1975; Coppel *et al.* 1983). S-antigen genes of several isolates of *P. falciparum* have now been sequenced. A basic structure for S-antigens has been described (Cowman *et al.* 1985), based on available sequence data, where the central portion of the protein is composed of tandemly repeating oligopeptides flanked by unique sequences. The unique sequences are relatively conserved between different isolates whereas the tandem repeats are very different. For

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example (Cowman *et al.* 1985), the FC27 isolate from Papua New Guinea (PNG) has approximately 100 repeats of the 11 amino acid sequence Ala-Lys-Ala-Ser-Gln-Gly-Gly-Leu-Glu-Asp-Pro, whereas the NF7 isolate from Ghana has 43 repeats of an 8 amino acid sequence Ala-Leu-Lys-Ser-Asp-Glu-Ala-Glu. There are two distinct versions of the NF7 octapeptide repeat due to a single base change in the 24 nucleotide repeating units resulting in a Leu to Arg change. Most of the natural antibody response in humans appears to be directed towards epitopes encoded by these repeat sequences.

Variation in the composition and number of amino acids in the repeats accounts for the serological diversity of S-antigens. Size polymorphisms result from variations in numbers of repeats and the number of constituent amino acids within a repeat. A mechanism for generation of diverse S-antigens appears to be a process of spreading point mutations, deletions and insertions throughout the repeat units. The function of the S-antigen system is unknown. The extreme serological diversity of S-antigens suggests a role in immune evasion. It is also possible that the conserved sequences of S-antigen may have a function. S-antigens may be targets of host protective immune responses, as a monoclonal antibody directed against the repeats of the FC27 S-antigen inhibits the growth of the homologous strain *in vitro* (Saul *et al.* 1984). Epidemiological studies (Wilson *et al.* 1975; Forsyth *et al.* 1988*b*) have also shown that individuals infected with a particular S-antigen serotype are unlikely to be reinfected with the same serotype.

3. S-antigens as serotypic markers

S-antigens are useful serotypic markers with which to analyse the transmission dynamics of sub-populations of *P. falciparum* for a number of reasons. First, as suggested by Wilson (1980), they are serologically diverse in nature but are stable characteristics of an isolate, persisting unchanged after numerous *in vitro* and *in vivo* passages. S-antigen genes behave as multiple alleles of a single locus. Thus the S-antigen system differs from the variant surface glycoprotein (VSG) of *Trypanosoma brucei*, as a cloned population of trypanosomes switches from one VSG to another (Borst & Cross 1982). Secondly, now that the molecular basis for S-antigen diversity is understood, highly specific serotyping reagents have been prepared by immunizing rabbits with isolate-specific repeat sequences of S-antigens (Saint *et al.* 1987). Thirdly, because S-antigens are found in circulation of *P. falciparum*-infected individuals they can readily be serotyped in small quantities of sera by highly sensitive enzyme-linked immunosorbent assays (ELISAS) employing antisera to isolate-specific repeat sequences of different S-antigens (Forsyth *et al.* 1988*a*). This antigen-detection method of serotyping is very convenient for sero-epidemiological investigations where large numbers of samples must be screened, as serum samples can be stored. In contrast, immunofluorescence serotyping assays require immediate processing of samples and time-consuming *in vitro* culture of parasites where polymorphic antigens to be serotyped are expressed at the schizont stage (McBride *et al.* 1985).

4. Transmission dynamics of S-antigen serotypes

The transmission dynamics of S-antigen serotypes have been examined in Madang, PNG by using antisera to isolate-specific repeat sequences of S-antigens in antigen-detection ELISAS (Forsyth *et al.* 1988*a*, *b*). A number of interesting findings have been reported highlighting the complexities of malaria transmission when parasite sub-populations are defined.

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(*a*) *Stability of three S-antigen serotypes in Madang parasite populations*

The distribution of three S-antigen serotypes associated with acute *P. falciparum* infections in children less than 15 years of age was assessed over a three-year period to determine the relative stability of S-antigen phenotypes in Madang parasite populations (Forsyth *et al.* 1988*a*). The frequency of distribution of the K1 and FC27 S-antigen serotypes did not vary significantly in the study population from 1984 to 1986. In contrast the NF7 S-antigen serotype was not found during this three-year period. Isolates of the FC27 S-antigen serotype were first identified in Madang in 1979 and of the NF7 S-antigen serotype in 1981. Thus it would appear that the FC27 S-antigen serotype has remained a stable phenotype in Madang parasite populations for at least eight years, whereas the NF7 serotype has not. The apparent loss of this serotype from Madang parasite populations may be a result of selection pressure by the immune response of the community to this serotype. Genetic polymorphisms in the host (e.g. red-cell polymorphisms) may also exert selection pressure if the S-antigen serves some essential function to the parasite.

(*b*) *Small-area variation in prevalence of an S-antigen serotype*

The transmission dynamics of the FC27 S-antigen serotype of *P. falciparum* have been examined in village communities surrounding Madang (Forsyth *et al.* 1988*b*). Cross-sectional analysis of the prevalence of a blood slide positive for *P. falciparum* infection and of circulating FC27 S-antigen in twelve villages showed marked village-based variation in the distribution of this serotype but not in slide positivity. Even neighbouring villages 2–5 km apart were shown to have a similar prevalence of *P. falciparum* infection but significantly different prevalence of the FC27 S-antigen. The small-area variation in transmission of the FC27 S-antigen serotype observed in this study is consistent with entomological data from Madang. Using mark–release–recapture experiments Charlwood *et al.* (1986) showed that the majority of anopheline vectors recaptured after release returned to the same village for subsequent blood meals. Limited mixing of mosquito populations between neighbouring villages would restrict transmission of serotypes and account for the localized nature of the transmission of the FC27 S-antigen serotype observed. Migration of residents between villages related by linguistic, marriage and agricultural ties is therefore likely to be more important in altering patterns of transmission of serotypes in Madang.

(*c*) *Periodic nature of transmission of S-antigen serotypes*

Longitudinal village-based malariometric studies have shown that the prevalence of the FC27 S-antigen serotype of *P. falciparum* not only varies among villages at a point in time but also within a village over time (Forsyth *et al.* 1988*b*). Similarly, analysis of the S-antigen serotypes associated with acute *P. falciparum* infections in residents from a single village community monitored for two years showed that the occurrence of subjects positive for the FC27 S-antigen were clustered in time (figure 1). There were two periods, differing in duration, in which the FC27 S-antigen was associated with acute infections in residents from Erima village. During the first two months of the study six residents had detectable FC27 S-antigenaemia. No further positives were detected for the next ten months but a prolonged period of transmission of this serotype occurred from month 13 to month 21, with an average of 1.6 subjects with FC27 S-antigenaemia out of 18 with a blood slide positive for *P. falciparum* infection detected per month. During the last three months of the study no further positives

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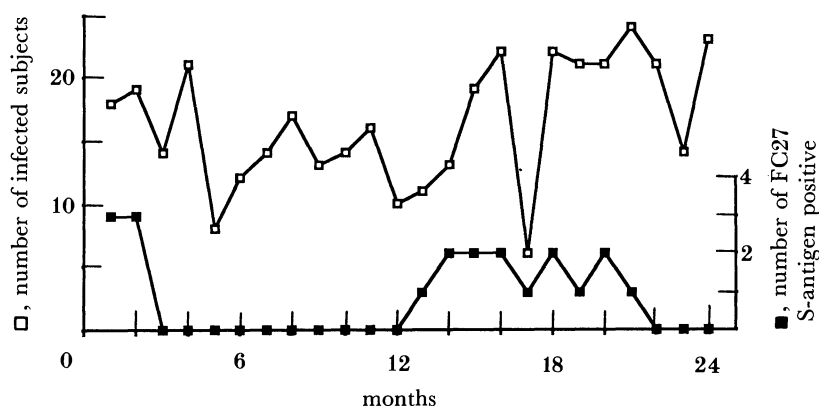

FIGURE 1. Longitudinal analysis of the number of subjects from Erima village, Madang, presenting to the local aid post with symptoms of malaria who had a blood slide positive for *P. falciparum* infection and circulating FC27 S-antigen over a two-year period. Open squares indicate the number of subjects with a blood slide positive for *P. falciparum* infection and closed squares indicate the number of subjects with detectable circulating FC27 S-antigen. Sera were analysed by ELISA by using rabbit antisera to the isolate-specific repeat sequence of the FC27 S-antigen.

were found. These data are consistent with the hypothesis that the transmission of the sub-population of *P. falciparum* parasites of the FC27 S-antigen serotype is periodic in village communities surrounding Madang.

Figure 2 shows a schematic representation of the periodic nature of the transmission of S-antigen serotypes of *P. falciparum* in a village community. From malariometric data described previously (Forsyth *et al.* 1988*b*) the prevalence of *P. falciparum* infection is generally greater than 25% in Madang villages. At any point in time this prevalence figure represents the sum of different S-antigen serotypes in the village. There would generally be more than one S-antigen serotype being transmitted in the village at a given time. Data from the longitudinal village-based studies indicate that transmission of individual S-antigen serotypes (designated A, B, C and D in figure 2) would occur in waves. These have been drawn as symmetrical curves but they may well be asymmetrical. The time interval for which individual serotypes A, B, C and D are maintained in the community is likely to vary. For example, transmission of the FC27 S-antigen serotype of *P. falciparum* was found to occur for at least nine months in the village of Erima (figure 1). Elimination of a serotype from parasite populations within a community is presumably the result of serotype-specific immunity. Measurement of IgG antibody responses to the repeat sequence of the FC27 S-antigen showed that there was a marked age-dependent acquisition of antibodies to this S-antigen serotype, and that recent transmission of the serotype in a village induced specific IgG antibody production in village residents and in particular in children less than 15 years of age (Forsyth *et al.* 1988*b*). Thus the proportion of children seropositive and the persistence of this serotype-specific immunity in children may be important factors controlling the maintenance and re-introduction of transmission of a particular S-antigen serotype in a community. Despite this serological data it is not clear whether immunity to a particular S-antigen serotype is protective against infection with this serotype. As genes for the other polymorphic asexual blood-stage antigens are on different chromosomes (Kemp *et al.* 1987), undoubtedly parasites of a particular S-antigen serotype will be heterogeneous with respect to some of these antigens. Thus if immune

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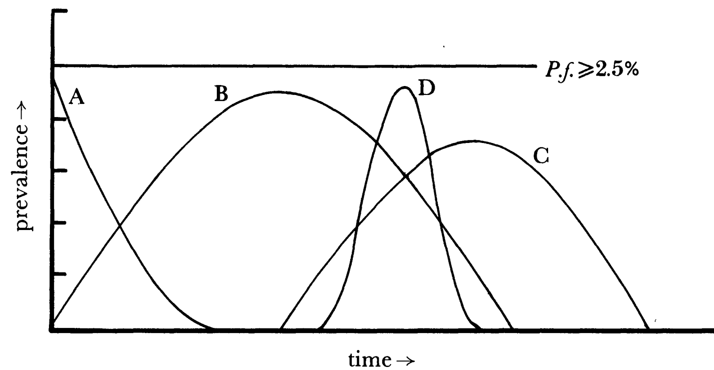

FIGURE 2. Schematic representation of the periodic nature of transmission of S-antigen serotypes designated A, B, C, and D in a village community in Madang, Papua New Guinea.

responses to such antigens have any protective effect, transmission of a particular S-antigen serotype will vary independently of anti-S-antigen responses.

(*d*) *Size heterogeneity of S-antigens*

Analysis of the relative molecular mass of S-antigens from culture supernatants of isolates of *P. falciparum* grown *in vitro* and from sera of infected subjects have shown that S-antigens display size heterogeneity (Anders *et al.* 1983; Winchell *et al.* 1984; Howard *et al.* 1986; Forsyth *et al.* 1988*a*). Size heterogeneity of the same as well as different serotypes has been reported (Saint *et al.* 1987). Molecular mass characterization of the FC27 S-antigen serotype on a large number of samples from Madang (Forsyth *et al.* 1988*a*) and PNG in general (R. F. Anders, unpublished observations) has shown that this serotype consistently has a molecular mass of 200 kDa. This result was somewhat surprising given that the molecular structure of S-antigens consists of a large central block of tandemly repeated amino acid sequences (Cowman *et al.* 1985). It is difficult to envisage why the number of amino acid repeats of the FC27 S-antigen serotype should be conserved. However, the lack of size heterogeneity of this S-antigen serotype suggests that there are some selective forces to maintain its size in PNG parasite populations. The prevalence of red-cell polymorphisms in the host population (Cattani *et al.* 1986) may be such a force if the S-antigen plays a role in red-cell invasion.

5. CONCLUSIONS

The S-antigen system has proved to be a useful serotypic marker to describe the transmission dynamics of a sub-population of *P. falciparum* parasites in Madang, PNG. Data from transmission studies have shown that two S-antigen phenotypes differ in stability in parasite populations of Madang. In addition, marked village-based variation in prevalence of an S-antigen serotype was observed to occur among villages in Madang at a point in time and within a village over time. The small-area variation in prevalence of S-antigen serotypes and the periodic nature of transmission of these serotypes within the village observed illustrate the complexities of malaria transmission. Heterogeneity in prevalence of serotypes among villages may have to be considered in the design of malaria vaccine trials if serotypes vary, for example, in relative virulence and transmission efficiency. With a more extensive collection of serotyping reagents to S-antigens and other polymorphic antigens from a given endemic area we may be better able to elucidate the natural history of malaria infection.

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Discussion

G. A. T. Targett (*London School of Hygiene and Tropical Medicine, London, U.K.*). The repeat sequences in S-antigens are quite different. Dr Forsyth has also shown that one serotype (FC27) occurred in three successive years in one part of Madang, and suggests that transmission is localized and FC27 is a stable serotype. Is there any evidence that, in a particular region, repeat sequences of the S-antigens and of other polymorphic antigens are similar, thus allowing the types of cross-reactivities proposed by Anders (1986)?

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Karen P. Forsyth. There is evidence of cross-reactivity between the repeat sequence of the FC27 S-antigen and the ring-infected erythrocyte surface antigen of *P. falciparum*.

D. J. Conway (*Department of Zoology, University of Edinburgh, U.K.*). If one considers the small spatial area (i.e. a village) in which frequency shift of the antigenic marker has been detected over time, and the fact that the antibody response to particular S-antigens is probably not effective in mediating parasite killing, perhaps it is small-area genetic drift that accounts for random fluctuations in the frequency of this marker, rather than any selection pressure from the immune response. Would Dr Forsyth's results allow us to distinguish between these two possible mechanisms?

Karen P. Forsyth. The fluctuations observed in the prevalence of the FC27 S-antigen cannot be due to genetic drift because the serotyping reagent used in these studies would detect any antigen that contained only a few copies of the 11 amino acid repeat sequence characteristic of the FC27 S-antigen. Thus the zero prevalence of this antigen in some villages must be due to replacement by other non-cross-reactive S-antigen serotypes. I have therefore hypothesized that serotype-specific immunity may generate the periodic transmission of the FC27 S-antigen serotype observed in village communities in Madang. Whether it is immunity to the S-antigen *per se* or some other antigen associated with it (e.g. on the same chromosome) is unclear. This hypothesis needs to be tested.

R. M. May, F.R.S. (*Department of Biology, University of Princeton, U.S.A.*). An implicit assumption in much work on the effects of frequency-dependent selection is that any polymorphisms so maintained will be steady, with gene frequencies changing over time only in response to mutation, drift, or other stochastic events. However, recent studies have suggested not only that interactions between hosts and pathogens are likely to maintain polymorphisms, but also that these polymorphisms may be steady or cyclic, or even fluctuating chaotically (Hamilton 1980; May & Anderson 1983). That is, the population genetics of host–pathogen associations can easily produce periodic, or even apparently random, fluctuations in gene frequencies (without any appeal to stochastic effects). I wonder if the temporal and spatial variations you have observed for *Plasmodium falciparum* may be caused, if only in part, by such deterministic mechanisms.

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Karen P. Forsyth. Yes it is possible that the observed fluctuations could be due to deterministic rather than stochastic mechanisms. However, the system has not yet been examined in this way.

D. A. J. Tyrrell, F.R.S. (*MRC Common Cold Unit, Salisbury, U.K*). This paper emphasizes an important aspect of vaccine development, namely that one needs not only an immunologically active product but also enough understanding of the biology and ecology of the pathogen to know how to use it. In the case of malaria it is hard enough to get a product, but Dr Forsyth's paper suggests that her research, which might be regarded as esoteric, is actually equally difficult and just as important if vaccines are to be given clinical trials and deployed in the community.

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