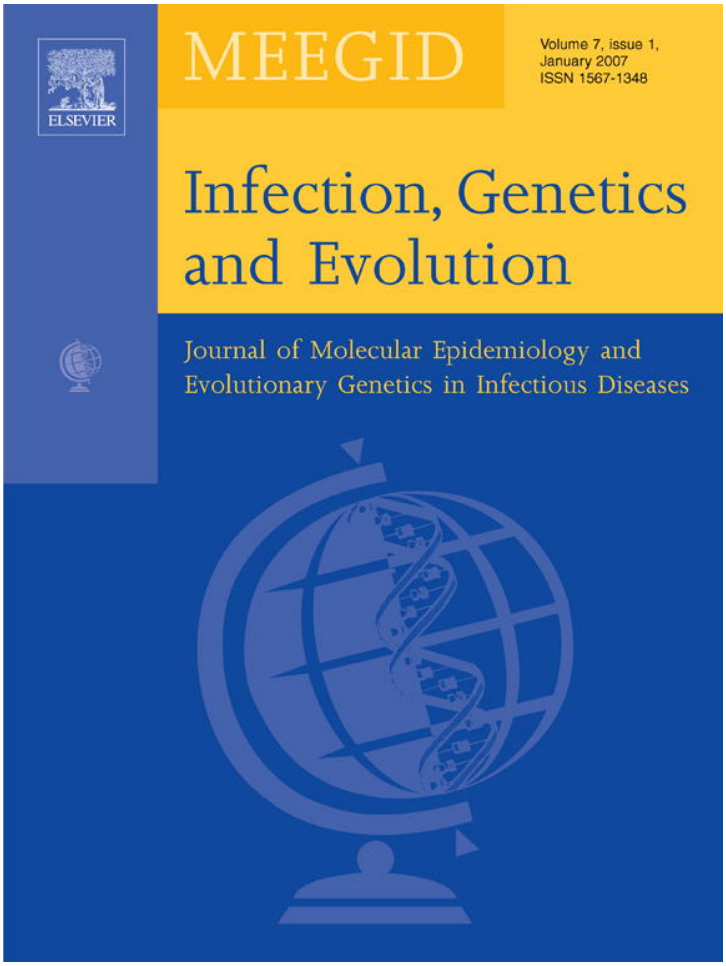

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Effect of the malaria vaccine Combination B on merozoite surface antigen 2 diversity

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Abstract

Extensive genetic polymorphism is generally found in *Plasmodium falciparum* surface antigens. This poses a considerable obstacle to the development of a malaria vaccine. In order to assess possible effects of a polymorphic vaccine, we have analyzed the genetic diversity of parasites collected in the course of a phase 2b field trial of the blood stage vaccine Combination B in Papua New Guinea. The full-length 3D7 allele of the merozoite surface protein 2 (MSP2) was included in Combination B as one of three subunits. Vaccinees had a lower prevalence of parasites carrying a 3D7-type allele (corresponding to that in the vaccine) and selection appeared to favour the alternative FC27-type alleles resulting in a higher incidence of morbid episodes associated with FC27-type parasites. We sequenced MSP2 alleles detected in study participants after vaccination to identify breakthrough genotypes. Extensive genetic diversity of MSP2 was observed in both the repetitive and family-specific domains, but alleles occurring in vaccine recipients were no different from those found in placebo recipients. A phylogenetic analysis showed no clustering of 3D7-type breakthrough infections from vaccine recipients. The repeat unit present in the vaccine molecule occurred in a number of alleles from the trial area and was also observed in vaccinated individuals. Thus the anti-repeat immune response did not lead to elimination of parasites carrying the same repeat unit. We conclude that the conserved epitopes in the family-specific domain were the most important determinants of the vaccine effect against new 3D7-type infections and that the hypervariable domains were not subject to selective effects of the vaccine.

Keywords: *Plasmodium falciparum*; Malaria vaccine; Breakthrough infection; Merozoite surface protein 2; Genetic diversity; Phylogenetic analysis

1. Introduction

Plasmodium falciparum surface proteins, and in particular those coating the invasive merozoite stage, are considered prime candidates for vaccine development. Merozoite surface proteins are accessible to the immune system between the rupture of a schizont-infected erythrocyte and reinvasion. Antigens located on the merozoite surface are generally well recognized by the immune system, but unfortunately these antigens also exhibit extensive polymorphism. Antigenic diversity is thought to help the parasite in escaping human immune defences (Anders, 1986).

Extensive genetic polymorphism poses considerable obstacles to vaccine design. If only a single allele of a polymorphic antigen is used as a malaria vaccine, the protection achieved might be directed only against the same or similar variants. The merozoite surface protein 2 (MSP2) of *P. falciparum*, which constitutes a major component of the surface coat of the merozoite, is an example of a highly polymorphic antigen that has been used for vaccination. The recombinant, full length MSP2 molecule was one component of the Combination B subunit vaccine along with MSP1 (190LCS.T3), and the Ring-infected Erythrocyte Surface Antigen (RESA). In 1998 Combination B was tested in a randomised, 4-armed placebo-controlled, double-blind Phase I/IIb trial (natural challenge) in 120 Papua New Guinean children aged 5–9 years. The subgroup of vaccinated children that were not treated with the anti-malarial sulfadoxine-pyrimethamine prior to vaccination, had on average 62% lower parasite densities than controls (Genton et al., 2002).

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A single allele of the highly polymorphic MSP2, deriving from the 3D7 strain, was included in the Combination B vaccine. The major portion of MSP2 is polymorphic, only the N- and C-terminal domains are conserved. A dimorphic region flanking a repetitive domain identifies the two allelic families of MSP2, the 3D7-type and FC27-type alleles. The units of tandem repeats vary considerably in length and sequence between different *msp2* alleles. Difference in repeat copy number causes extensive length polymorphism, which forms the basis of various *msp2* genotyping schemes.

Genotyping all blood samples collected during the trial at fortnightly intervals over 18 weeks revealed that the vaccine exhibited specificity for infections belonging to the 3D7 allelic family. This efficacy against 3D7 parasites was not evident in blood samples collected from morbid episodes during an extended 1-year follow-up. However, during the follow-up there was a higher incidence of clinical episodes with FC27-type parasites in vaccinated children than in placebo recipients. These were the first reports of a selective effect exerted by vaccination with a polymorphic malaria vaccine (Genton et al., 2002; Felger et al., 2003).

Combination B, as most other malaria vaccines currently in development, was not expected to provide sterilizing immunity. Such imperfect vaccines may select for specific breakthrough parasites, comprising variants not cleared by vaccine-induced immunity. While genotyping of all samples from the vaccine trial had shown that the 3D7 vaccine differentially affected the alleles of the FC27 and 3D7 family (Genton et al., 2002), the vaccine effect on individual 3D7-type MSP2 alleles from subsequent infections has not yet been examined. To monitor the impact of the imperfect MSP2 vaccine subunit of Combination B on the subsequent genetic diversity of MSP2, we have now sequenced *msp2* alleles from both breakthrough and placebo group infections. Phylogenetic sequence analysis was applied to identify characteristics of genotypes resistant to vaccine effects. Such analysis has been postulated to be of great relevance, since partially effective vaccines could potentially select for more virulent pathogens (Gandon et al., 2001).

2. Materials and methods

2.1. Study population

One hundred and twenty children aged 5–9 years from the Wosera area of Papua New Guinea were recruited for a phase I/IIb field trial of Combination B, starting in February 1998. The study was designed as a four-armed placebo controlled trial with one half of the children being pre-treated with SP at baseline. Venous or finger prick blood samples were collected at baseline and during eight consecutive cross-sectional surveys as described by Genton et al. (2002). During a 1-year morbidity follow-up finger prick blood samples were taken from all children reporting with a history of fever in the last 3 days.

2.2. Sample collection

From the 120 children enrolled in the trial, 1079 blood samples were collected at baseline and during the 18 weeks follow-up period. Isocode stix dip sticks (Schleicher and Schuell) were used for transport and storage of blood pellets after removal of serum. All samples were analyzed by PCR, and 257 of these were found to be positive for *P. falciparum*. In addition, 449 blood samples were collected from those 120 children during the 1-year morbidity follow-up (296 samples from self-reported case detection at the health center and 153 samples from community-based case detection through weekly visits by village reporters). From these 449 morbidity follow-up samples, 202 were positive for *P. falciparum* by PCR.

2.3. Genotyping

Isolation of *P. falciparum* DNA and *msp2* genotyping was performed as previously described (Felger et al., 1994; Felger and Beck, 2002). The variable central part of the *P. falciparum msp2* gene was amplified by PCR. Subsequent restriction digests produced a genotype-specific RFLP pattern for each different parasite clone in a blood sample. PCR-RFLP genotyping distinguished 39 *msp2* alleles in 257 parasite-positive blood samples of the trial. During the following year of morbidity follow-up, three additional *msp2* alleles were detected in morbid episodes. This amounts to 42 *msp2* alleles (eight of the FC27 family, 34 of the 3D7 family) present in the study area.

2.4. Sequencing

During the entire study we sequenced 32/34 different 3D7-type *msp2* alleles identified by PCR-RFLP. Alleles from Single infections were chosen for analysis. Two alleles only occurred in multiple infections also harbouring other alleles of the same allelic family. Thus direct sequencing was not possible for 2/34 alleles. The nested *msp2* PCR product was directly sequenced using both PCR primers and two internal sequencing primers (5′-CAGTTTGTTCGGCTGTTGGA-3′ and 5′-CTGAAGAGGTACTGGTAGA-3′). The sequencing reaction was performed with Big Dye sequencing reagents (Applied Biosystems) according to the supplier's instructions and loaded to an ABI PRISM 310 genetic analyzer. The ABI Sequence Navigator program was used for sequence analysis. Sequences were submitted to Genbank under the accession numbers: U07001, AY534507, U07009, U16840, U16842, DQ162622, DQ168572, DQ166534, DQ185319, AJ318755, AJ318753, AJ318752, AJ318754, DQ166535, DQ174442, DQ166536, DQ158904, DQ185320, DQ168571, DQ166545, DQ166537, DQ166538, DQ166546, DQ166539, DQ166540, DQ166541, DQ166542, DQ166543, DQ166544, DQ171731, DQ171732, M73810.

2.5. Phylogenetic analysis

For sequence alignment the ClustalX program (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) was used. Phylogenetic analysis was performed with PHYLIP (Phylogeny Inference Package) version 3.6.a3 (Felsenstein, 2002).

Distance and parsimony methods were chosen to calculate the fractions of sites that differ between MSP2 alleles. Trees were constructed by the Neighbor Joining as well as by the parsimony algorithm using 1000 bootstrap replicates. Phylogenetic trees were viewed by the Tree View program version 1.5.2 (Page, 1996).

2.6. Definition

A breakthrough infection was defined as a new infection which occurred from week 8 onwards in children immunized with Combination B, and which has not been present in the blood sample of the same individual at baseline and at weeks 4 and 6. Immunizations were carried out at baseline and at week 4.

3. Results

3.1. Genetic diversity of 3D7-type msp2 alleles

Thirty-four distinct genotypes of the 3D7 family were identified by PCR-RFLP in the course of the entire study. Two genotypes could not be directly sequenced due to high multiplicity of infection in the respective blood samples. Seven genotypes were detected in baseline samples only, or persisted from baseline onwards but did not occur in new infections.

We analyzed 37 breakthrough parasite clones and 41 control clones from placebo recipients. Twelve RFLP genotypes occurred in both, vaccine and placebo, 7 only in vaccine and 6 only in placebo. There is no evidence that a subset of genotypes was selectively eliminated by vaccination. This might reflect chance effects in transmission.

3.2. MSP2 gene trees

Phylogenetic analysis of genes with intragenic repeats is difficult. Sequence alignment of 3D7-type *msp2* alleles was problematic because repeat units of the various alleles differed in sequence and lengths and were often scrambled. The repeat region includes tandem and scrambled repeats and is defined on nucleotide sequence level by regular spacing of thymidines, giving rise to solely (XXT) codons (Felger et al., 1997). This previous observation was confirmed by all new *msp2* alleles sequenced in this study. Fig. 1 shows an alignment of the polymorphic domains of 32 3D7-type MSP2 alleles detected in the trial area. For comparison, the 3D7 vaccine molecule is added to the alignment, despite the fact that this variant was not present in the study area. The repeats are followed by a non-repetitive but polymorphic region of variable length, which reveals several clusters of similar sequences. Downstream of this variable region all 3D7-type *msp2* alleles contain a poly-threonine (T) stretch, also varying in lengths, which represents on the nucleotide sequence level in fact another tandem repeat. Further downstream of the poly-threonine stretch follows the so-called family-specific domain of 90 residues that in parts is well conserved with the exception of a region of microheterogeneity (starting at position 14 after poly-T). This region of microheterogeneity revealed 10 SNPs within a stretch of 25 residues, and synthetic peptides representing a part of this region bound to human red blood cells and inhibited parasite invasion of erythrocytes (Ocampo et al., 2000). Just upstream of the repeats, a second small region of microheterogeneity within the family-specific domain is located, spanning six residues of which only the serine at position 3 is conserved.

In order to quantify the sequence similarity between each allele and the 3D7 vaccine molecule, we performed a distance analysis and applied the Neighbour Joining algorithm. First the analysis was performed with the entire polymorphic region between the N- and C-terminal constant domains. The resulting MSP2 gene tree is shown in Fig. 2a. The vaccine molecule 3D7 (accession number M28891) was chosen as outgroup when drawing the tree. We found that breakthrough infections were not clustered in any branch of the tree, and that alleles not found in breakthrough infections were distributed over all clusters. The same analysis was performed with alignments from which increasing portions of the variable regions had been removed. Fig. 2b shows the MSP2 gene tree obtained after the (XXT)n repeat region had been removed. Both trees were consistent in their key finding, that breakthrough infections found in vaccinated individuals showed no greater distance to the vaccine molecule than alleles from the placebo group. In addition to the distance method for constructing a gene tree, we also applied the maximum parsimony algorithm. Trees obtained were similar with the major clusters maintained (data not shown). Breakthrough alleles were again evenly spread over all clusters of the tree.

Because the region of microheterogeneity located downstream of the poly-T Stretch and spanning 25 residues was implied in competitive invasion inhibition (Ocampo et al., 2000), we also analyzed this region in greater detail. For Fig. 2c this region of 25 residues was aligned together with a stretch of six residues preceding the tandem repeats and also displaying sequence heterogeneity. The gene tree showed that breakthrough infections were not clustered and did not reveal less similarity to the 3D7 vaccine molecule than sequences found in the placebo group. Thus, vaccination obviously did not select for alternative variants. However, the considerable number of variants detected in our samples suggests that this region is nevertheless under selection.

3.3. Repeats of breakthrough infections

The intragenic repeats of 3D7-type alleles are mainly responsible for length polymorphism. Some alleles share the same repeat units, but vary in copy number of repeats (Felger et al., 1997). We were interested in how far the 3D7 repeats, which had been found to be immunogenic in the Combination B vaccine trial (Flück et al., 2004), might have cross-protected vaccinated children against becoming infected with an allele carrying the same repeat unit. We found that, while the 3D7 allele (accession number M28891) representing the vaccine molecule, was not present in the study area, its 4-mer repeat glycine-glycine-serine-alanine (GGSA) was present in three

Fig. 1. Sequence alignment of the 3D7-MSP2 vaccine molecule and 32 3D7-type MSP2 alleles that were detected in children participating in the Combination B vaccine trial in PNG. Names of sequences correspond to Genbank accession numbers. Stretches of sequence microheterogeneity are bold.

alleles from the trial area. One allele harbouring a GGSA repeat (accession number DQ166535) was detected in two vaccinated children. The same 4-mer motif was also represented in another, larger repeat unit present in four additional alleles (DQ174442, DQ171732, DQ166539, DQ166543), three of which were found in vaccinees.

3.4. Sequence fidelity and persistence in time of msp2 alleles in the study area

At the site of the vaccine trial, in the Wosera area in PNG, *msp2* diversity has been studied 6 years prior to the trial by using the same genotyping technique. In this previous cross-sectional survey in 1992, 38 different *msp2* alleles of both families were detected in two villages (Felger et al., 1994). When we compared the nucleotide sequence and frequencies of RFLP-genotypes, we found that the most frequent genotypes in 1992 were still frequent 6 years later, and that some alleles had been maintained without a single point mutation. Alleles of low allelic frequency seem to fluctuate and were mostly new.

Because PCR-RFLP detects length polymorphism and mutations at restriction sites, it does not reveal all sequence diversity present. In order to establish sequence fidelity within a RFLP genotype, we chose the most frequent allele, KF1916, for a detailed sequencing analysis. Seven nested *msp2* PCR products, all classified as KF1916 genotype by PCR-RFLP, were directly sequenced. These KF1916 sequences were aligned together with the original KF1916 sequence from Genbank (accession number M73810) that derived from a PNG isolate adapted to culture in the 1980s (Marshall et al., 1992). Three KF1916 sequences from the 1992 survey were also added to the alignment. Fig. 3 shows that KF1916 is well conserved,

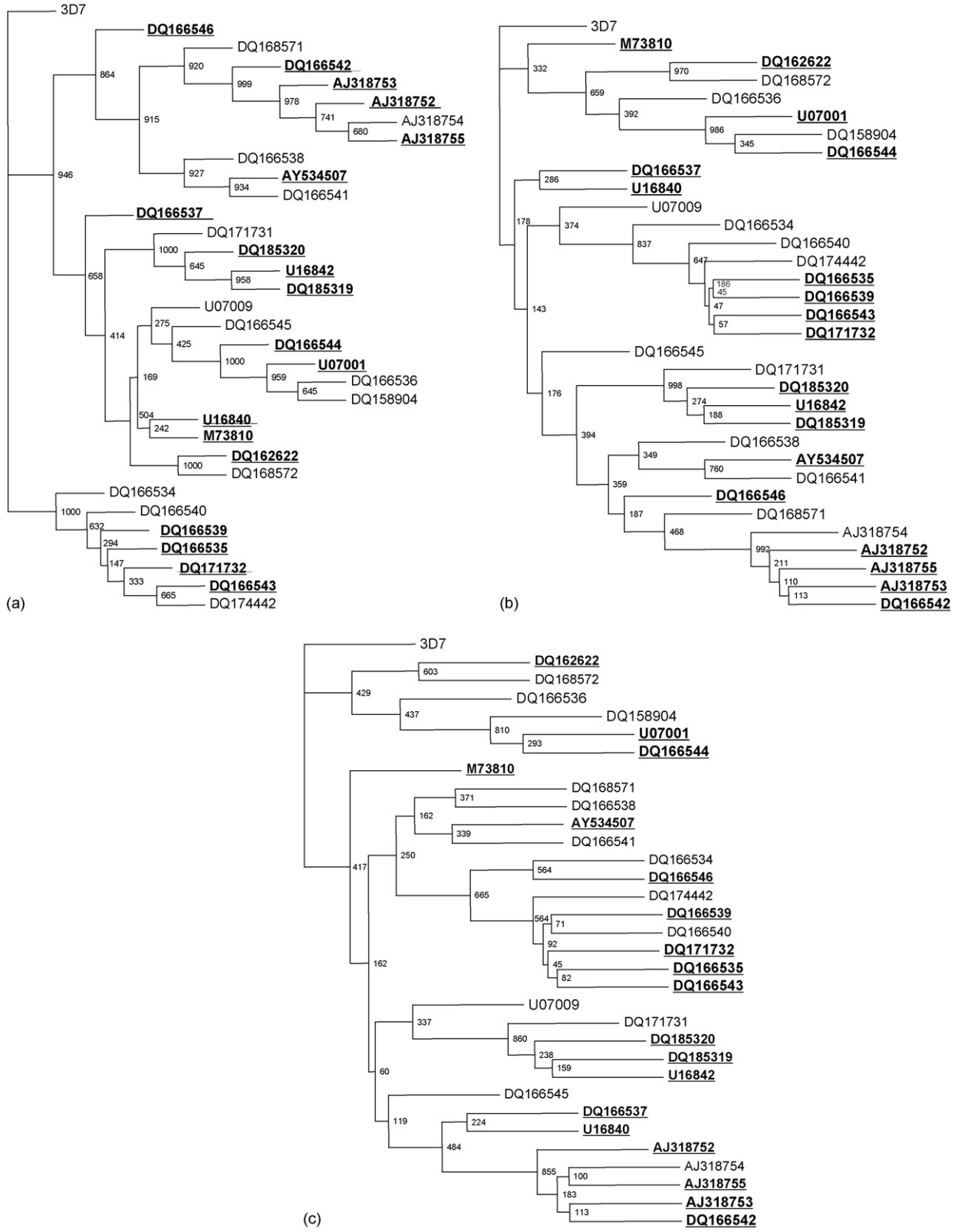

Fig. 2. Phylogenetic analysis of 3D7-type *msp2* alleles detected in children from the Combination B trial. Sequences occurring in breakthrough infections (new infections after week 8 post-vaccination) of vaccinated children are underlined. The phylogenetic tree was built using the Neighbor Joining method with 1000 bootstrap replicates. The tree was drawn with the vaccine molecule 3D7 as outgroup. The scale bar indicates sequence distance. (a) *msp2* gene tree obtained from an alignment of the entire variable region including repeats. (b) *msp2* gene tree based on 3D7-type *msp2* sequences from which the repeats had been deleted. (c) *msp2* gene tree based on regions of microheterogeneity flanking the repetitive domain.

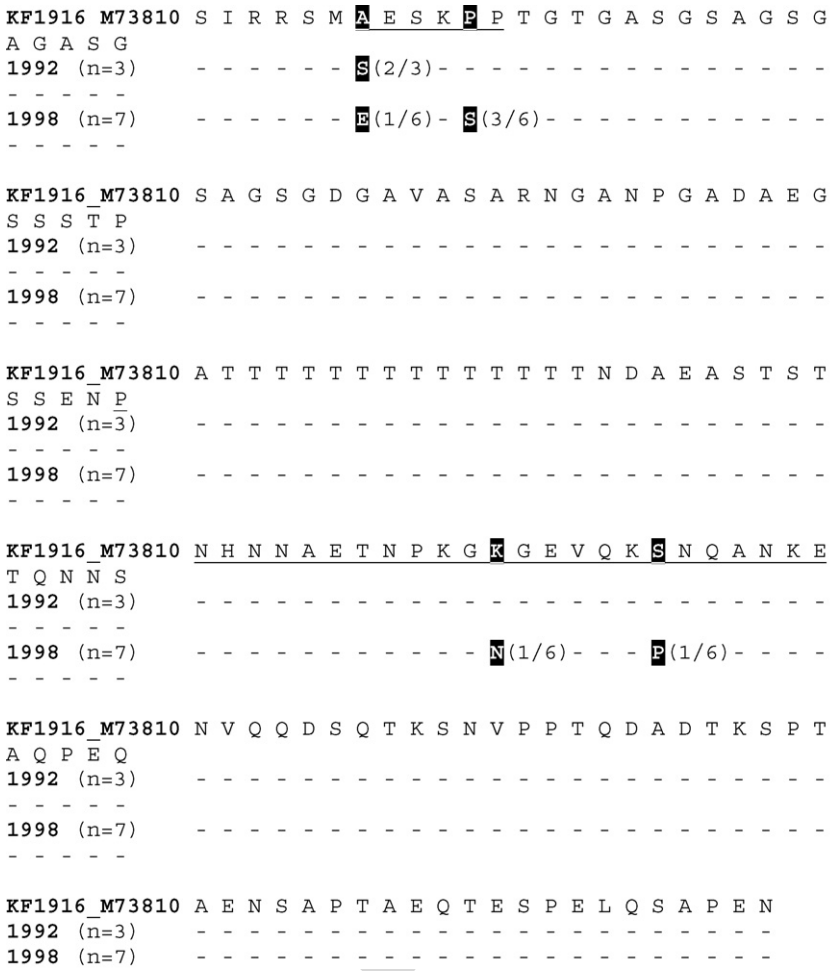

Fig. 3. Sequence fidelity of the PCR-RFLP genotype KF1916 of MSP2 over a period of 20 years. Isolates were collected in Papua New Guinea in 1992 and 1998 and compared to the KF1916 sequence submitted to Genbank that derived from an isolate collected in the 1980s. The polymorphic central part of *msp2* corresponding to the nested PCR product is shown. Underlined residues correspond to the two regions of microheterogeneity where SNPs are frequent.

mutations did not accumulate over time, and SNPs were found at an average frequency of one per clone sequenced. The repetitive domain and most part of the family-specific domain were totally conserved whereas SNPs were only detected in the two regions of microheterogeneity. From these data we concluded that PCR-RFLP genotypes are stable over time.

In summary, our results showed a high amount of sequence heterogeneity at the trial site. Allelic diversity in *msp2* was not restricted to the actual repetitive domain alone. High diversity was also found in a considerable part of the dimorphic or family-specific domain. Strictly conserved within all 3D7-type MSP2 alleles was a stretch of 53 residues upstream of the C-terminal constant domain. Specifying the boundaries of strictly conserved dimorphic domains has implications for design of other MSP2 vaccine molecules. We showed that in the Combination B vaccine trial, the diverse domains seem to have not contributed to vaccine efficacy, because breakthrough infections in vaccinated individuals were independent of similarity with the 3D7 vaccine molecule. This suggests that the well-conserved epitopes in the family-specific domain must have been the important determinants of the vaccine effect against 3D7-type infections.

4. Discussion

Antigenic diversity in *P. falciparum* represents a significant challenge for the development of a malaria vaccine. As polymorphism is prevalent in most *P. falciparum* antigens, it is unrealistic to expect complete parasite clearance in vaccinated individuals. Not even natural immunity prevents infection entirely, and sterilizing immunity is never achieved in individuals from endemic areas. Most current efforts in malaria vaccine development consider partially effective vaccines and combination of multiple subunits consisting of several candidate antigens or of several variants of a polymorphic vaccine molecule is the currently favoured strategy (Mahanty et al., 2003).

Little evidence from field data is available on selective effects in malaria vaccine trials. Despite the extensive polymorphism of MSP2, the MSP2 allele of the 3D7 strain was included as a subunit in the Combination B malaria vaccine. Thus, it is likely that escape mutants emerge in the population by filling ecological niches emptied by variants eliminated by vaccine-induced immunity. Our previously published genotyping results from the Combination B trial

had shown selection acting on the level of allelic families (Genton et al., 2002). Now we have investigated whether a vaccine effect is evident also on the level of individual alleles. The impact of the imperfect 3D7-MSP2 vaccine on breakthrough infections occurring in vaccinated children was assessed by sequence analysis of all *msp2* alleles detected during the trial.

One objective of our analysis was to verify that genotypes detected by PCR-RFPL were stable in time, and thus matched sequences submitted to GenBank. To date, little is known on the temporal and geographic genetic differentiation among parasite populations. The concept of microepidemics is still under debate. The 3D7-type allele (KF1916) was the most frequent in the Wosera area, both in 1992 and 1998, and showed little variation in frequency. We have so far not been able to analyse the sequence conservation over time in the less frequent RFLP-defined genotypes. More longitudinal analyses similar to those of KF1916 are needed to understand the dynamics of *P. falciparum* population structure.

4.1. Msp2 repeat units as smoke screen epitopes

The function of intragenic tandem repeats in plasmodial surface antigens remains obscure. It has been speculated that the arrays of repeats represent ''smoke screen'' epitopes, which divert the immune system from protective responses by directing the response to irrelevant repetitive epitopes (Anders, 1986; Kemp et al., 1987). It has been proposed that repeats are immunodominant, but even so induce only non-neutralizing antibodies by crosslinking hapten-specific surface immunoglobulin on B cells thus providing a thymus-independent activation with no memory elicited (Schofield, 1991). It is generally assumed that levels of anti-repeat antibodies are not correlated with protection, but this has not yet been shown conclusively for MSP2. Our data cannot provide much evidence to either support or reject this hypothesis, mainly because the $(Gly\text{-}Gly\text{-}Ser\text{-}Ala)_5$ repeat of the vaccine molecule was found only rarely in alleles in the study area. Nevertheless, we inspected the anti-$(Gly\text{-}Gly\text{-}Ser\text{-}Ala)_5$ response at week 12 post-vaccination in the few vaccinated individuals infected with a parasite displaying the (Gly-Gly-Ser-Ala) motif, either as tandem repeat or as part of a larger repeat unit. From those individuals antibody titres were available in the serological database of Flück et al. (2004). Antibody titres were either not raised at week 12 compared to baseline values, or only a minor increase was observed (data not shown). Thus, it remains unclear whether infection by these genotypes could have been prevented if an anti-$(Gly\text{-}Gly\text{-}Ser\text{-}Ala)_5$ response had been induced in these vaccinated children.

4.2. Variable non-repetitive domain

After deleting the repeat regions from all sequences of the alignment of 3D7-type MSP2 alleles, a region of about 50 residues of the dimorphic domain proved totally conserved. This region is located adjacent to the C-terminal conserved region. Further upstream, flanking the repeats, highly polymorphic non-repetitive blocks are found characterized by an accumulation of point mutations. We have tested particularly whether these regions of microheterogeneity were subject to selection in vaccinated individuals. We found that sequence similarity of this SNP-rich region to the vaccine molecule did not affect a genotype's presence or absence in the immunized group. Therefore, it has to be assumed that responses elicited against these regions were not protective. It follows that the 50-residues-long totally conserved dimorphic region might have been responsible for the selective effect acting on the level of the allelic family.

We assume that the regions of microheterogeneity were immunogenic, because several studies have mapped immunogenicity to these regions. Immunization with a short peptide including a part of the C-terminal region of microheterogeneity (peptide 40 in Jones et al., 1992) elicited IFA-positive antibodies. Lawrence et al. (2000) have mapped linear antibody epitopes within MSP2 after vaccinating a human volunteer with the 3D7 variant of MSP2, corresponding to the 3D7 component of Combination B. The same analysis was performed after immunizing mice with the same molecule. Both experiments showed in parallel that the regions of local microheterogeneity were found to be the major targets of antibody response in the family-specific domain of MSP2. This hypervariable region downstream of the poly-threonine stretch revealed 10 sites of non-synonymous mutations clustered in a stretch of 25 amino acids (underlined in Fig. 3). It is exactly this block that seems to play an important role in merozoite invasion of human red blood cells. Ocampo et al. (2000) have identified an MSP2 peptide with high specific binding to human erythrocytes, which is identical with this block. The peptide also inhibited in vitro parasite invasion by up to 95%. It remains unclear how a possible function in invasion can be reconciled with the hypervariability we have documented in our small sample size.

4.3. Conserved regions within the family-specific domain

The Combination B vaccine was effective in reducing parasite densities, yet the effect was incomplete. Despite vaccination, some 3D7-type infections could establish themselves in immunized children but the vaccine effect may have lead to faster elimination and thus to the reduced prevalence of 3D7-type parasites observed in the trial (Genton et al., 2002). In search of new improved vaccine formulations the question arises which domain of MSP2 could have caused the selective effect? The 3D7 family-specific domain contains 50 residues of invariant sequence. This is a likely candidate to account for selection observed on the level of the allelic family. We have previously shown that antibody levels against the recombinant 3D7 family-specific domain and against the 3D7 repeats were significantly higher in vaccinated children (Flück et al., 2004). However, our phylogenetic analysis does not allow us to pinpoint the active component of the 3D7 subunit vaccine; it can only indicate whether a polymorphic region is selected and thus subject to protective antibodies. As with naturally induced immune responses, responses elicited by vaccination are directed against many different epitopes, only a fraction of

which might lead to protection. We assume that an antibody response against both the repeats and regions of microheterogeneity was elicited, but did not protect the vaccinated children against new infections. This does, however, not exclude activity of these antibodies against high parasite densities.

This analysis does not take into account the pre-existing acquired immunity to 3D7 genotypes nor the possibility that an ongoing 3D7-type infection inhibits a newly occurring infection of the same allelic family via within-host competition (e.g., for resources). The extent of competitive interactions in multiple clone infections is only recently being studied (de Roode et al., 2004). It is unclear whether such conditions can be ignored in the analysis of selectivity.

5. Conclusion

The vaccine trial has shown that the 3D7-MSP2 component had some efficacy, though this was imperfect and numerous breakthrough infections occurred. When analyzing the breakthrough genotypes, we found no evidence that responses against the Gly-Gly-Ser-Ala repeats and hypervariable stretches might have protected against new infection by similar variants. We conclude that a response against the conserved Stretches within the family-specific dimorphic domain is more likely to account for the MSP2 family-specific selective effect seen in the Combination B trial.

The occurrence of replacement by FC27-type infections in vaccinees in the Combination B trial, leading to increased morbidity, confirms that selective effects of imperfect polymorphic malaria vaccines are of real concern. As more results from other and bigger trials of polymorphic vaccines become available, it will become clear whether such selective effects are a general side effect of vaccine interventions. There is a clear need to include several variants of a polymorphic vaccine candidate in a single vaccine formulation.

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