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AGE-DEPENDENT CARRIAGE OF MULTIPLE *PLASMODIUM FALCIPARUM* MEROZOITE SURFACE ANTIGEN-2 ALLELES IN ASYMPTOMATIC MALARIA INFECTIONS

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Abstract. Genetic diversity of the merozoite surface antigen-2 gene of the human malaria parasite *Plasmodium falciparum* has been analyzed in a Senegalese village where malaria is holoendemic. A cross-sectional survey of 65 residents was performed in 1992 during the high transmission season. *Plasmodium falciparum* was detected both by microscopy (77% positive samples) and DNA amplification using a single (29% or 38% positive samples, depending on the primers used) or nested polymerase chain reaction (PCR) (78% positive samples). The overlap between the positive nested PCR and microscopic examination was not complete. The PCR fragments were analyzed for size polymorphism on agarose gels, and were subsequently assigned to the major allelic families 3D7 or FC27 by hybridization with family-specific probes. Both allelic families were found, with a slightly higher prevalence for FC27. Chimeric alleles that failed to hybridize under stringent conditions to the reference probes were also observed. Some were typed using a novel PCR approach, using hybrid pairs of primers, consisting of a family-specific sense oligonucleotide combined with an antisense oligonucleotide specific for the other family. Combining typing techniques, 82% of the positive PCR results yielded more than one band. Both the overall number of fragments and the number of allelic types per carrier were markedly reduced around the age of 15 years. The number of DNA fragments decreased abruptly from an average of four per carrier before the age of 15 years to an average of two in individuals more than 15 years of age. Similarly, the number of individuals carrying more than one allelic type decreased with age, with a cutoff at the age of 15 years. This parallels the observed decrease in prevalence and parasite density in this village. There was, however, no age-dependent carriage of any particular allele, with the various alleles being detected in all age groups. The results, therefore, indicate that acquiring anti-parasite immunity not only results in decreasing parasite load, but also in decreasing the complexity of the infections.

Immunity to malaria is acquired slowly after many infections and requires frequent boosting. Experimental infections in humans have shown that the immunity raised to one strain of *Plasmodium falciparum* is largely inefficient against challenge with a heterologous strain.[1] It is believed that the slow acquisition of protection is mainly a consequence of the long period required to achieve exposure to a large repertoire of serotypes.[2] However, some long-lasting, low-grade *P. falciparum* infections, such as those induced by an attenuated strain in *Saimiri* monkeys, elicit a protective immunity that is not strain-specific.[3] An understanding of the parameters governing the acquisition of protective immunity requires a better knowledge of host/parasite interactions. This includes evaluation of the extent of local parasite diversity and elucidation of the role that such a polymorphism plays in the human immune responses to those parasites. A major characteristic of human malaria parasites is their genetic diversity.[4] Allelic polymorphism,[4] recombination,[5,6] and chromosome rearrangements,[7] as well as antigenic variation,[8–10] result in an extensive antigenic diversity. This is further amplified through the independent chromosome reassortments at meiosis, which occur prior to any new infection. Polymorphism of field isolates collected from patients[11–13] has been reported for many characteristics, such as enzymes, proteins, antigens, and drug sensitivity. For example, a total of 39 serotypes have been defined for the *P. falciparum* merozoite surface protein-1 (MSP-1) using monoclonal antibodies.[13] Recently, the polymerase chain reaction (PCR) has been used to analyze the genomic diversity of field isolates.[14] To date, a few loci have been studied from infected blood samples collected in endemic areas.[15–19] However, the results obtained do not reflect the actual local diversity of malaria parasites because the majority of the field isolates analyzed to date have been collected in large geographic regions and from symptomatic malaria patients. In a cross-sectional survey in Papua New Guinea, parasites carried by asymptomatic individuals have been typed for merozoite surface antigen-2 (MSA-2) and nine new alleles were found.[18] Recently, Marshall and others reported a molecular analysis of MSA-2 alleles collected in a geographically restricted area of Irian Jaya and again showed sequence heterogeneity.[19]

To study the parameters influencing protection against malaria, an epidemiologic survey has been undertaken in a Senegalese village called Dielmo, where malaria is holoendemic.[20,21] The work described here is part of a cross-sectional survey done in Dielmo in which the parasites infecting asymptomatic individuals during the transmission season have been typed by nested PCR for the MSA-2 locus. In parallel, the humoral and cellular immune responses of the individuals to MSA-2 have been studied (Sarthou JL and others, unpublished data). The MSA-2 gene codes for a merozoite surface polymorphic glycoprotein[22–24] that is a putative vaccine candidate. The sequencing of DNA has shown that the N- and C-terminal domains are well conserved, whereas the central region varies in number, length, and type of repetitive sequences.[25–27] All alleles studied so far can be grouped into two main allelic families, 3D7/ICI and FC27.[25,26] Up to 22 different allelic forms have been found.[19,25,26,28] Within each family, alleles differ by the type

and number of repeats, deletions, insertions, or nucleotide substitutions.[27] We report here an analysis of this gene in parasites collected from 65 asymptomatic carriers using a single and a nested PCR. Both allelic families were detected. At least 23 different alleles were observed. Multiple band patterns were observed in many individuals. A marked age dependence was observed for the number of fragments and alleles detected in these asymptomatic donors.

MATERIALS AND METHODS

Study site, blood donors, and parasites. The cross-sectional survey was conducted in Dielmo, a village of 250 inhabitants, located in the Fatick region, 280 km southeast of Dakar. The rainy season occurs between July and October, but due to the presence of a permanent stream, malaria transmission is perennial.[20] Sixty-five blood samples were collected from asymptomatic residents (38 males and 27 females, 1–84 years of age) during the transmission season (from July 21 to August 26, 1992), with the informed consent of the individuals or their parents. The blood analyzed was from venous blood samples collected for a joint study of parasite polymorphism and immune response. The red blood cell pellet was frozen after separation from white blood cells. Slides were prepared, stained with Giemsa, and examined by microscopy for at least 200 oil immersion fields. Parasites were quantified as described elsewhere.[20] Fourteen samples, in which there was a divergence in positivity for the PCR and microscopy were re-examined for 300–400 fields before these slides were definitively characterized as negative or positive.

Extraction of DNA. The DNA was extracted from saponin-lysed, thawed red blood cell pellets by treatment at 37°C for 1 hr with 5 mg/ml of proteinase K (Boehringer Mannheim, Meylan, France) in 50 mM Tris, pH 8.0, 10 mM EDTA, 0.15 mM NaCl, 0.5% sodium dodecyl sulfate (SDS), 1% Triton-X100, followed by two extractions with phenol/chloroform/isoamyl alcohol (25:24:1). The DNA was then precipitated with ethanol and resuspended into 50–70 μl of 50 mM Tris, pH 8.0, 0.1 mM EDTA, i.e., approximately half of the original volume of red blood cell pellet.

Polymerase chain reaction amplification. The sequences of the primers used are indicated in the legend to Figure 1. Amplifications were done using a Hybaid thermal reactor (Cera-Labo, Ecquevilly, France) in a final volume of 50 μl. Reactions contained 1 μl of DNA, 200 μM of each deoxynucleotide (Pharmacia, Saint-Quentin-Yvelines, France), 1 μM of each primer, 12 mM $MgCl_2$, and 2.5 units of *Taq* polymerase (Promega, Madison, WI) in the reaction buffer supplied by the manufacturer. Samples were denatured in the PCR mixture for 5 min at 94°C and amplified for 30 cycles (2 sec at 94°C, 1.5 min at 55°C, and 2 min at 72°C). For the nested PCR, the primary PCR was done using primers 1 + 4 and consisted of a denaturation step of 5 min at 94°C followed by 25 PCR cycles performed using the same conditions as above. The second reaction was performed for 25 cycles using 1 μl of the primary PCR mixture and primers 2 + 3, A1 + B2, or B1 + A2.

Agarose gel electrophoresis and Southern blot analysis. A 10-μl aliquot from each PCR was loaded onto a 1% agarose gel in the presence of 0.5 μg/ml of ethidium bromide,

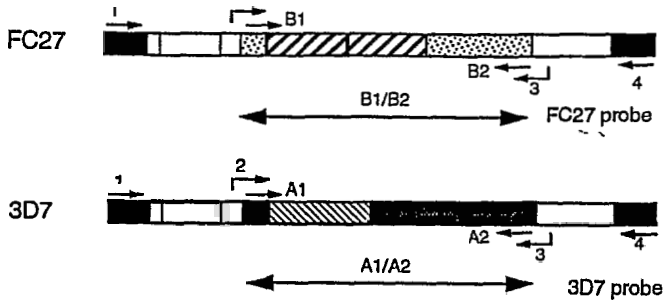

FIGURE 1. Schematic representation of the FC27 and 3D7 merozoite surface antigen-2 alleles of *Plasmodium falciparum* modified from Smythe and others.[28] The black regions on the left and the right represent the domains coding for the signal and anchor sequence, respectively. The unfilled regions represent conserved regions. The central polymorphic domain contains family-specific unique sequences located in a 5′ and 3′ orientation (depicted as the dotted region), as well as a family-specific central repeated region (depicted as the hatched region). Specific symbols have been used for the 3D7 and the FC27 families. The relative position and orientation of the primers are represented by arrows. The FC27 and 3D7 probes were obtained by amplification of reference alleles using primers B1 + B2 and A1 + A2, respectively. The conserved oligonucleotide sequences are derived from Symthe and others.[25] 1 = 5′ (position 1-30) ATGAAGGTAATTAAAACATTGTCTATTATA; 4 = 3′ (position 760-790) ATATGGCAAAAGATAAAACAAGTGTTGCTG; 2 = 5′ (position 79-114) AACGAATTCATAAACAATGCTTATAATATGAGT; 3 = 3′ (position 662-697) GATGAATTCTAGAACCATGCATATGTCCATGTT. The allele-specific primers were derived from Fenton and others.[27] A1 = 5′ (position 130-157) GCAGAAAGTAAGCCTCCTACTGGTGCT; A2 = 3′ (position 366-391) GATTTGTTTCGGCATTATTATGA; B1 = 5′ (position 129-154) GCAAATGAAGGTTCTAATACTAATAG; B2 = 3′ (position 504-530) GCTTTGGGTCCTTCTTCAGTTGATTCA.

subjected to electrophoresis, and visualized with ultraviolet light. Following photographing of the gel, the DNA was transferred to a Hybond N nylon membrane (Amersham, Les Ulis, France) as recommended by the manufacturer. Hybridization was done with ^{32}P-radiolabeled PCR probe (FC27- or 3D7-specific probes amplified from reference lines using primers B1 + B2 or A1 + A2, respectively) for 18 hr at 65°C in 6× SSC (20× SSC = 3M NaCl, 0.3M sodium citrate), 2.5% nonfat milk, 0.1% SDS, and 100 μg/ml of herring sperm DNA. Filters were washed several times at 65°C with decreasing concentrations of SSC (6× SSC, 2× SSC, 0.5× SSC, and 0.1× SSC). Membranes were exposed at −70°C using X-OMAT film (Eastman Kodak, Rochester, NY) after the 2× SSC washes (nonstingent conditions), the 0.5× SSC washes (medium stringency), or the 0.1× SSC washes (high stringency). Under the latter conditions, only those molecules presenting a high degree of homology with the probe remain hybridized. The same filter was used for successive probing with the allele-specific 3D7 and FC27 probes. The first probe was removed from the filter following the hybridization instructions provided by the manufacturer.

Statistical analysis. To measure the association between two ordinal variables, we used the Spearman rank-order correlation coefficient. To test the differences in the number of bands among age classes, the Kruskal-Wallis one-way analysis of variance by ranks was used. The chi-square test was

TABLE 1

Comparison between the numbers of Plasmodium falciparum*–positive individuals as determined by microscopic examination of thick blood smears or by the polymerase chain reactions (PCR) using various primer combinations*

Microscopic results	Primers 1 + 4		Primers 2 + 3		Nested PCR	
	Negative	Positive	Negative	Positive	Negative	Positive
Positive (n = 50)	26	24	31	19	3	47
Negative (n = 15)	14	1	15	0	11	4
Total (n = 65)	40	25	46	19	14	51

used to test the significance of different frequencies between two groups.

RESULTS

Comparison between microscopic examination and the single or nested PCR. Fifty of the 65 samples analyzed (77%) were positive for *P. falciparum* asexual parasites and/or gametocytes by microscopic examination. Parasitemia ranged from approximately 1 to 15,000 parasites/μl. To type these parasites, several PCRs were done on all samples. The primers used were derived from highly conserved regions (Figure 1), assuming that the sequences would also be conserved in the parasites circulating in Dielmo. Single PCRs were done using either primers 1 + 4, aimed at amplifying the entire coding region or primers 2 + 3, located close to the borders of the central polymorphic domain (Figure 1). Neither reaction was sensitive enough. Therefore, we developed a nested PCR consisting of a first reaction using primers 1 + 4, followed by a secondary reaction using primers 2 + 3.

In Table 1, the sensitivity of the three PCRs are compared with that of microscopic examination. Using PCR 1 + 4, 24 of the 50 thick blood smear–positive samples and one microscopically negative sample yielded a PCR product. In the reactions using primers 2 + 3, amplification was obtained for only 19 blood samples, all of which were positive microscopically. The nested PCR resulted in MSA-2 amplification in 47 of 50 microscopically positive specimens; three samples with positive results on slides remained negative with the nested PCR (one of those yielded PCR products in both single PCRs, but was negative with the nested PCR for an unknown reason; one had a few gametocytes and one had one gametocyte, the species of which could not be clearly identified). Four microscopically negative samples yielded a PCR product. These are interpreted as harboring very low parasite densities (< 1 parasite/μl). Three were from adults and one was from a three-year-old child who had been treated for a symptomatic malaria attack two weeks previously and who subsequently had another symptomatic malaria episode 1.5 months later. This child probably harbored a low gametocytemia at the time of blood collection. Overall, the nested PCR was as sensitive as microscopic examination, and significantly more sensitive than the single PCR using primers 1 + 4 (38% positive samples) or primers 2 + 3 (29%).

Size polymorphism and hybridization. The products resulting from the nested PCR were typed for size polymorphism on agarose gels. A typical example is shown in Figure 2A. The fragments ranged in size between 450 and 650 basepairs. Size polymorphism was relatively restricted, since nine distinct fragments sizes could be visualized on the gels used. Multiple bands were observed in 82% of the amplifications. To assign each fragment to one of the two major allelic families (FC27 or 3D7), hybridizations using family-specific probes were carried out. Each gel was probed sequentially with an allele-specific probe under nonstringent and stringent conditions, as described by Mercereau-Puijalon and others.[16] Hybridization showed that many samples contained both allelic families. As indicated in Table 2, 49% of the fragments hybridized to FC27 and 399% to 3D7; 12% of the fragments hybridized under nonstringent conditions, but not stringent conditions, and have been grouped as unknown, probably due to disruption of an unstable hybridization product formed between the probe and a chimeric gene. Therefore, these were typed by the hybrid PCR.

Allele typing with nested allele-specific primers (hybrid PCR). Nineteen fragments remained unassigned by hybridization using family-specific probes. To identify these, another strategy was used, assuming that they were recombinant alleles or mosaic genes as described by Marshall and others.[29] After the primary PCR using the conserved primers 1 + 4, a second set of allele-specific primers was used. These primers were derived from the variable regions of the MSA-2 gene, as depicted in Figure 1. Two 3D7-specific and two FC27-specific primers were prepared to perform a hybrid-specific PCR in which one 3D7-specific primer was used in combination with one FC27-specific primer. The results of a typical experiment are shown in Figures 2B and C. The DNAs in lanes a and c contained a FC27-3D7 hybrid, whereas those in lanes j, k, and l contained a 3D7-FC27 hybrid. In lane f, both hybrids were detected.

Table 2 indicates that some PCR fragments not assigned to a specific family by hybridization could be identified as chimeric forms of MSA-2 using the hybrid PCR: seven were 5′ 3D7-FC27 3′ and two were 5′ FC27-3D7 3′. One sample, recorded as containing a single unknown fragment, generated two hybrid products (one was the 5′ 3D7-FC27 3′ and the other was the 5′ FC27-3D7 3′) of similar size, indicating that it did contain two distinct hybrid genes. Among the six isolates containing hybrid MSA-2 genes, only two also had 3D7 and FC27 types. Even by using this approach, 11 fragments still remained untyped (an example is shown in Figure 2, lane d).

Allelic diversity and prevalence. The results summarized in Table 2 indicate that both allelic families were present in the village. Of 160 fragments, 78 were typed as FC27, 63 as 3D7, nine as hybrid genes (seven 3D7-FC27 and two FC27-3D7) resulting from a single cross-over as described by Marshall and others,[29] and 11 remain undefined alleles, most probably complex chimeras. Five distinct FC27 alleles and six different 3D7 alleles that differed by size were detected. Six distinct 5′ 3D7-FC27 3′ hybrids and one 5′ FC27-3D7 3′ hybrids were observed. Among the unassigned alleles, five different sizes were also noted. A total of 23 distinct alleles, defined by size and type, were therefore observed. The various alleles were distributed throughout all the age ranges studied. There was no preferential carriage of any allelic type in any age group. Carriage of hybrid

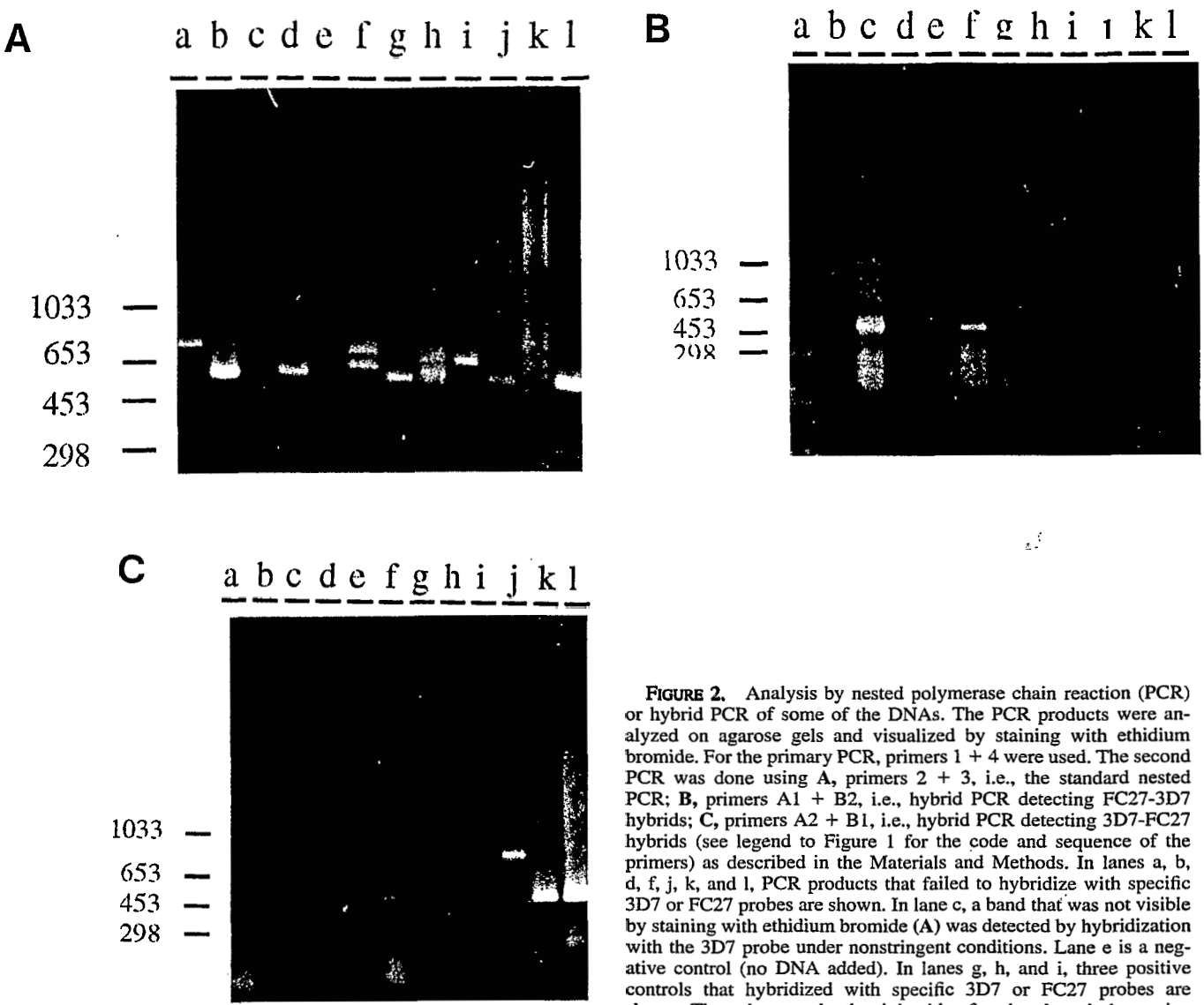

FIGURE 2. Analysis by nested polymerase chain reaction (PCR) or hybrid PCR of some of the DNAs. The PCR products were analyzed on agarose gels and visualized by staining with ethidium bromide. For the primary PCR, primers 1 + 4 were used. The second PCR was done using **A**, primers 2 + 3, i.e., the standard nested PCR; **B**, primers A1 + B2, i.e., hybrid PCR detecting FC27-3D7 hybrids; **C**, primers A2 + B1, i.e., hybrid PCR detecting 3D7-FC27 hybrids (see legend to Figure 1 for the code and sequence of the primers) as described in the Materials and Methods. In lanes a, b, d, f, j, k, and l, PCR products that failed to hybridize with specific 3D7 or FC27 probes are shown. In lane c, a band that was not visible by staining with ethidium bromide (**A**) was detected by hybridization with the 3D7 probe under nonstringent conditions. Lane e is a negative control (no DNA added). In lanes g, h, and i, three positive controls that hybridized with specific 3D7 or FC27 probes are shown. The values on the the right side of each gel are in basepairs.

TABLE 2

*Distribution of various merozoite surface antigen-2 (MSA-2) alleles by age groups**

Age (years)	No. PCR-positive	No. (%) of fragments 3D7	No. (%) of fragments FC27	No. (%) of fragments unknown	Hybrid PCR
0–4	6	10 (37)	14 (52)	3 (11)	1 3D7-FC27 2 NI
5–9	13	20 (39)	24 (47)	7 (14)	5 3D7-FC27 2 FC27-3D7 1 NI
10–14	5	10 (42)	14 (58)	0 (0)	–
15–19	6	5 (38)	7 (54)	1 (8)	1 3D7-FC27
20–39	12	10 (36)	11 (39)	7 (25)	7 NI
≥40	9	8 (47)	8 (47)	1 (6)	1 NI
Total no. of fragments	160	63 (39)	78 (49)	19 (12)	7 3D7-FC27 2 FC27-3D7 11 NI

* For each age group, the total no. of fragments hybridizing with the 3D7 or FC27 probe under stringent conditions are indicated. Fragments that failed to hybridize are recorded as unknown. The last column indicates the outcome of the hybrid polymerase chain reaction (PCR) typing. NI = nonidentified MS2-2 alleles; – = no hybrid bands were observed.

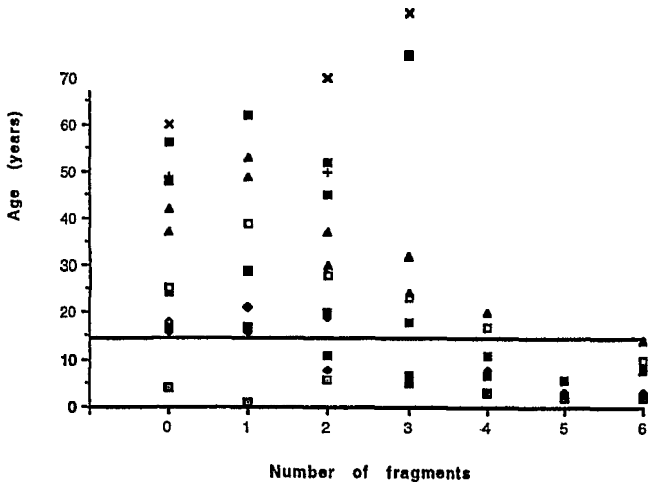

FIGURE 3. Age distribution of the number of fragments calculated from staining with ethidium bromide and hybridization patterns. The various DNA samples have been grouped according to the number of fragments generated in the nested polymerase chain reaction (0 to 6); each symbol represents an individual donor. The horizontal line delineates samples from individuals < 15 years old from those ≥ 15 years old.

genes or of unidentified alleles was not linked to concomitant carriage of the 3D7 and FC27 types.

Age-dependent multiple allele carriage. The number of fragments per parasitemic carrier was estimated by counting both the bands visualized by staining with ethidium bromide and those detected by hybridization (excluding the results of the hybrid PCR because it was not carried out on all samples). Hybridization was more sensitive than staining with ethidium bromide and sometimes identified additional bands. The number of bands detected by the approach used here was 0–6 per sample and multiple bands were found in 42 of the 51 nested PCR-positive samples. Importantly, the number of fragments generated was correlated with parasitemia (Spearman rank coefficient $\rho = 0.6$, $Z = 4.6$, $P = 0.001$). However, despite this general tendency, several samples with low parasite densities, as estimated by thick blood films, gave multiple bands, including the four thick blood smear–negative samples that yielded 2–5 fragments. Conversely, some samples with high parasite densities gave one or two band profiles. Figure 3 shows the distribution of the mean number of bands observed after the nested PCR as a function of age. Fourteen samples were negative in the nested PCR. As already noted above, 11 of these had been collected from microscopically negative individuals and three from individuals with positive slides. Interestingly, 13 of the 14 nested PCR–negative samples were from adults, and only one was from a four-year-old child, who had been treated for a symptomatic episode three weeks previously and did not have any malaria symptomatic attack for the next 4.5 months. As shown in Figure 3, nine DNAs yielded a single band; one of these was from a one-year-old child (presenting with no malaria-attributable fever in the preceding period, but with a malaria episode three weeks later) and the other eight were from adults (> 16 years of age). Among the 12 samples giving two bands, three were obtained from children and of the 12 giving three fragments, four were from children. All

TABLE 3

*Age distribution of the mean number of merozoite surface antigen-2 fragments per nested polymerase chain reaction (PCR)–positive individual**

Age (years)	No.	No. PCR-positive	Total no. of fragments	No. of fragments/ carrier	No. (%) of individuals >1 allelic type
0–4	7	6	27	4.5	5 (83)
5–9	13	13	51	3.9	11 (85)
10–14	5	5	24	4.8	4 (80)
15–19	9	6	13	2.2	1 (17)
20–39	15	12	28	2.3	4 (33)
≥40	16	9	17	1.9	1 (11)

* The no. of fragments was calculated from the bands observed on anagarose gel after staining with ethidium bromide and hybridization with family-specific probes. The allelic type was determined by hybridization with family-specific probes and/or a hybrid PCR.

the DNA preparations generating five or six fragments were from children, including children with low parasite densities. Therefore, there was a clear trend for complex multiple infections in children and for infections with a smaller number of distinct alleles in adults. This is summarized in Table 3, in which the mean number of bands carried per individual has been analyzed by age group. In the groups 0–4, 5–9, and 10–14 years of age, the mean number of fragments per infected individual was four (range 3.9–4.8), and in those greater than 15 years of age, this figure decreased significantly to two (range 1.9–2.3) (Kruskal-Wallis test $H = 28.2$, $P = 0.0001$).

In addition, the results of hybridization also indicated a marked age-dependent carriage of multiple allelic types. Bands were classified into four categories: 1) positive by hybridization under stringent conditions to 3D7, 2) positive by hybridization to FC27, 3) positive by the hybrid PCR method developed here, and 4) positive by hybridization under nonstringent conditions to 3D7 or FC27, but negative by hybrid PCR (unidentified samples in Table 2). These categories reflect major differences in the gene, and will be referred to as allelic types. Table 3 shows that the number of isolates in which more than one allelic type could be detected decreased with age. In children less than 15 years of age, a majority of the carriers harbored parasites identified as belonging to distinct types. In contrast, this was rare in individuals more than 15 years of age (22%) ($\chi^2 = 18.9$, $P < 0.0001$).

DISCUSSION

The PCR methodology has been used in laboratory or field studies for both parasite detection and analysis.[16–18,30–32] For most published PCR methods, the detection threshold for *P. falciparum* was approximately 10 parasites/μl.[32–34] Wataya and others[35] developed a simple and highly sensitive DNA diagnosis system using a nested PCR that detected a *P. falciparum* parasitemia of 0.0002–0.002% in field samples (i.e., approximately 8–80 parasites/μl). The nested PCR was shown to increase the sensitivity of detection 10-fold for *P. vivax* and *P. malariae* and 100-fold for *P. ovale* as compared with a single PCR.[33,34] In pilot experiments using laboratory strains, the minimum number of parasites that could be detected with the single PCR developed in our laboratory was approximately 25 parasites/μl (Contamin H and

others, unpublished data). However, the yield using field samples varied, with some samples being detected below this threshold, while others with significantly higher parasitemias could not be amplified using either PCR and in some cases, both individual PCRs. This problem was mostly overcome using a nested PCR, which allowed us to detect and type parasites even from microscopically negative carriers. Nevertheless, three DNA samples from parasite carriers identified by microscopy could not be amplified by the nested PCR (one of these is difficult to understand because this sample was positive using both single PCRs). A lack of concordance between microscopy and the PCR in the analysis of field samples has been reported for *P. vivax* by Kain and others[30] and for *P. falciparum*.[32,36] Various explanations can be proposed, including the presence of mutations preventing hybridization with the primers, a possible erroneous microscopic diagnosis, a technical error in collecting or processing samples, the presence of PCR inhibitors in DNA preparations, or degradation of parasite DNA during transportation from the site of collection to the laboratory.

Using the nested PCR, a total of 160 fragments were observed in 51 positive samples. The majority of these bands were confirmed as being amplification products because they hybridized to family-specific probes under nonstringent conditions. Twenty-three different alleles were detected. The polymorphism of this locus is most probably underestimated by the approach used here. It is likely that more subtle size differences could be visualized under conditions that allow better resolution and that additional chimeras would be identified by a systematic hybrid PCR on all samples. Furthermore, it is clear that some mutations should not be detected by hybridization because long probes rather than oligonucleotides were used. Using long probes presents the major advantage of allowing hybridization over large regions of the polymorphic domain and of estimating the extent of mismatches by increasing the stringency. However, their use does not allow detection of mutations that do not have a major impact on hybrid stability. An in-depth analysis of the polymorphism would require DNA sequencing of a large number of fragments and analysis of a larger number of parasite carriers. Therefore, the results reported here provide a rough estimate of the genetic diversity of this locus and indicate that there is a significant diversity. This contrasts with the relatively homogeneous sampling from Irian Jaya analyzed recently by Marshall and others.[19] In Dielmo, FC27-type alleles accounted for 49% of the fragments and 3D7-type alleles accounted for 39%. These figures differ from the higher prevalence of 3D7 parasites in countries such as Brazil,[12] Sudan,[37] Irian Jaya,[19] or The Gambia.[13] In Colombia, however, the proportion of the FC27 alleles was similar to that found in Dielmo.[17]

Multiple parasites genotypes in individuals living in endemic areas are frequently observed. Creasey and others[12] reported that in Zimbabwe, mixed infections represented 83% of the analyzed samples, and that these figures were 50% in Thailand and 30% in Brazil. Mixed parasite populations were also observed in Sudan[37] and in The Gambia.[38] Multiple bands were observed in 82% of the PCR-positive samples from Dielmo. It must be noted that the PCR conditions used here allowed amplification of single bands from cloned laboratory parasite lines and from many samples collected from individuals living in areas where malaria transmission is low, such as Dakar (Mercereau-Puijalon O, unpublished data). In Dielmo, up to six distinct fragments per carrier were observed using the nested PCR, with a mean of four per child and two per adults. This is significantly higher than those previously observed in other studies. Snewin and others studied 31 clinical isolates collected in Colombia and observed only two MSA-2 mixed infections generating two bands.[17] In The Gambia, isolates collected from malaria patients (mostly children) and containing a mean of two distinct parasite genotypes were detected by Conway and others[38] using monoclonal antibody typing or by Carter and McGregor[39] using isoenzymes. Our study differs from these in several respects. First, the parasites studied here were all collected from asymptomatic carriers who may harbor more antigenically complex infections than patients experiencing a clinical attack. Second, malaria transmission is intense in Dielmo and significantly higher than in The Gambia or in Colombia. During July and August 1992, the period in which the isolates studied had been collected, the mean number of *P. falciparum* infective bites/person/month were 29 and 15, respectively. The multiple carriage most probably reflects the frequency of infective bites received during this period by the inhabitants of Dielmo.

The most striking observation was that the mean number of distinct alleles harbored per individual decreased abruptly at the age of 15 years. In the younger age group (0–14), the number of MSA-2 fragments was four and in 80–85% of the cases corresponded to the presence of more than one allelic type. In the older age group, the mean number of fragments was approximately two, and a minority (11–33%) of the samples contained multiple allelic types. An age-dependent distribution of *P. falciparum* densities is a common observation in many endemic regions.[40] A marked decrease in parasite densities after 11–15 years of age is observed in regions of intense transmission, and clearly occurs in Dielmo.[21] This reflects the efficiency of the antiparasite immunity acquired at this stage of life. This immunity does not prevent new infections and is believed to be isolate-specific.[1,2] Little is known about the mechanisms involved in this immunity and their targets. The results reported here indicate that at the time at which an efficient, antiparasite immunity is observed, the complexity of the parasite population harbored by asymptomatic individuals decreases significantly. This suggests that the acquired antiparasite immunity is able to eliminate or control multiplication of a large number of the parasites that are inoculated, thereby not only reducing parasite loads but also parasite types. These conclusions, based on the study of a single marker, are consistent with those recently reported by Gupta and others.[41] We are now extending this analysis to other markers to determine more precisely the extent of genetic diversity of the local parasite populations and more importantly, to investigate whether the restricted number of allelic types in adults is specific for the MSA-2 locus or is also observed for other polymorphic loci coding for potentially important targets of protective immunity.

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