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Human Antibody Response to the Major Merozoite Surface Antigen of *Plasmodium falciparum* Is Strain Specific and Short-Lived

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The precursor of the major merozoite antigen of *Plasmodium falciparum*, gp190, is considered a candidate for inclusion in a malaria vaccine. This protein, which consists of conserved, dimorphic, and polymorphic sequences, is very immunogenic in humans. In a longitudinal study carried out with 94 inhabitants of a rural community in Mali, West Africa, we show that in this endemic area naturally acquired gp190-specific antibodies are predominantly directed against the dimorphic parts of one of the two main alleles of gp190. The presence of antibodies against these dimorphic regions correlates with the prevalence of the corresponding antigen in the infecting parasite population. Moreover, qualitative as well as quantitative differences were found in the time course of the humoral immune response to the dimorphic regions in adults and children, who differ in their susceptibility to malaria infection.

Immunity to malaria develops with age after repeated exposure to parasite infections. The nonsterile and short-lived character of such immunity and its specificity with respect to species, stage, and strain are well documented, although not sufficiently understood (14). A better description of the mechanisms involved would be useful for both vaccine development and the evaluation of malaria control programs.

Direct evidence for the role of humoral immunity in protection was provided by the observation that gamma globulins from immune adults dramatically accelerated the clearance of parasites when passively transferred to infected children (3). The blood stage antigens which induce such humoral immunity in adults have not yet been clearly identified. Data provided from different laboratories propose for this role, among others, the precursor of the major merozoite surface antigens of *Plasmodium falciparum*, gp190 (reviewed in reference 8). This protein, in fact, is highly immunogenic in humans, and immunization of monkeys with purified gp190 modifies the course of infection upon challenge. However, the assessment of the role of gp190 in eliciting a protective humoral immune response in humans has been complicated by the observation that parts of this antigen are polymorphic (13, 19). Furthermore, experimental vaccination trials with gp190 purified from parasite cultures suggest that the polymorphic parts should not be omitted if an evaluation of the protective properties of the immune response to gp190 is undertaken. Monkeys were protected to a higher extent against challenge with the homologous than with a heterologous strain (6, 18).

Analysis of genomic sequences of different parasite strains revealed a striking pattern of polymorphism of gp190: whereas some regions of the molecule are highly conserved among all strains, the largest part is dimorphic, leading to two main serotypes (19). Subserotypes were shown to be, in general, the result of two short sequences found in the N-terminal portion of the protein (2, 8, 10, 17).

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Previously, we were able to demonstrate that naturally acquired antibodies of immune individuals living in Burkina Faso were mainly directed against the dimorphic regions of both main alleles (15). Here we present a longitudinal survey performed in a malaria hyperendemic area of Mali with strong seasonality in transmission. The study was designed (i) to analyze the humoral immune response of adults and children to both main gp190 alleles in a different endemic area; (ii) to quantify the seasonal changes of anti-gp190 antibodies; and (iii) to identify the gp190 serotype of the parasites present in this area.

MATERIALS AND METHODS

Study area and study population. The village of Safo is situated 15 km northeast of Bamako, Mali, and inhabited by about 1,000 individuals living either in clusters of housings or in compounds scattered around the village. Nearly all villagers are farmers. Rainfalls are strongly seasonal in this area, starting usually in June and ending in October.

Health care, which was not available before the study, was provided free on all occasions when the staff visited the village and on request in between visits. Malaria detected in blood smears was not treated unless it caused febrile episodes.

Venous blood was withdrawn from 37 adults and 57 children on three occasions. The first blood samples were collected after the beginning of the rain season, 24 to 30 July 1988; the second samples were collected after the rains had ceased (24 to 30 November 1988); and the last samples were collected before the beginning of the following year's wet season (23 to 26 May 1989). Also, thick and thin smears of capillary blood were obtained from fingerpricks and stained with Giemsa. Parasitemia was evaluated by examining the proportion of infected erythrocytes in as many microscopic fields as necessary to count 300 leukocytes.

Entomological parameters. In October 1988 mosquitoes were collected in 24 houses, using spray sheets. Also, mosquitoes were hand caught from 48 individuals in different parts of the village during one nightly session. The biting

rates (bites per person per night) were 3.0 with the spray catch method and 11.5 with the hand catch method during October 1988. Collection and interpretation of the entomological data were performed by using World Health Organization recommendations (21, 22).

The presence of sporozoites in the mosquitoes was determined with an anti-CS protein monoclonal antibody (4). Of a total of 688 mosquitoes, 121 were positive in the test. The mean infectious bites per person per night was calculated by multiplying the biting rate by the sporozoite rate. At the end of the dry season, in May 1989, no infected mosquitoes were detected in the same houses.

Parasite culture and immunofluorescence. During the visit in November, blood from eight children with parasitemia exceeding 0.1% was taken into heparinized tubes (Vacutainer; Becton Dickinson). The blood was washed twice in RPMI (GIBCO) and cultured in RPMI–10% AB+ medium, using a candle jar (20). After 24 to 48 h, the presence of schizonts was assessed by analysis of Giemsa-stained blood films. Erythrocytes were washed twice in phosphate-buffered saline and diluted to 1% hematocrit; 25 μl was spotted onto six-well glass slides. The slides were air dried and fixed for 5 min in acetone. Immunofluorescence serotyping with gp190-specific monoclonal antibodies was carried out with these slides as described before (13).

Recombinant gp190 fragments. Expression of gp190 fusion proteins in *Escherichia coli* has been described before (15). In short, genomic DNA coding for gp190 of the MAD20 and K1 strains was cut by using naturally occurring restriction sites. The DNA fragments were inserted 5′ to the dihydrofolate reductase (DHFR) or chloramphenicol acetyltransferase (CAT) gene of vector pUH31-1. The resulting fusion proteins were used as antigens in immunoblot analysis.

To obtain pure products for enzyme-linked immunosorbent assay (ELISA), the 3′ ends of fragments M6 and M7 were converted into *Bgl*II sites by linker addition and inserted into vector p(His)$_6$-DHFR (7), using the *Bam*HI and *Bgl*II cloning sites, which leads to deletion of the DHFR coding region. Thus, a tail of six histidine residues was fused to the amino terminus of the fragments. The presence of the histidine tail allows the purification of recombinant proteins in a single step by nickel-chelate chromatography (7). Total bacterial protein was dissolved in 6 M guanidinium-hydrochloride (pH 8.0) and directly applied to the nickel column. Bound protein was eluted by lowering the pH stepwise to pH 4.0.

Immunoblotting. As described by Müller et al. (15), total bacterial lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose filters. Each filter displayed all 10 fusion proteins used in the study together with bacterial lysates containing CAT and DHFR as controls. For immunoblot analysis, the sera were diluted 1:100 and preadsorbed to nitrocellulose filters soaked with bacterial lysate.

ELISA. Each of the purified fragments was coated onto Falcon microtiter plates in 0.1 M $NaHCO_3$ by overnight incubation (100 ng of protein in 50 μl per well). Free binding sites were blocked overnight with 200 μl of 1% bovine serum albumin in the same buffer. After aspiration of the blocking buffer, the sera were added in quadruplicate at a dilution of 1:100 in phosphate-buffered saline–1% Tween 20. The sera were allowed to react for 2 hours with the antigen followed by 10 washings with phosphate-buffered saline–0.3% Tween 20. For the detection of bound antibodies, goat anti-human immunoglobulin serum conjugated to alkaline phosphatase (Promega) was used at a dilution of 1:7,500. After 1 h, the plates were washed as above and remaining phosphatase activity was revealed by addition of 50 μl of 10% diethanolamine–10 mM $MgCl_2$–1 mM 4-nitrophenylphosphate per well. The A_{405} was measured in an ELISA reader.

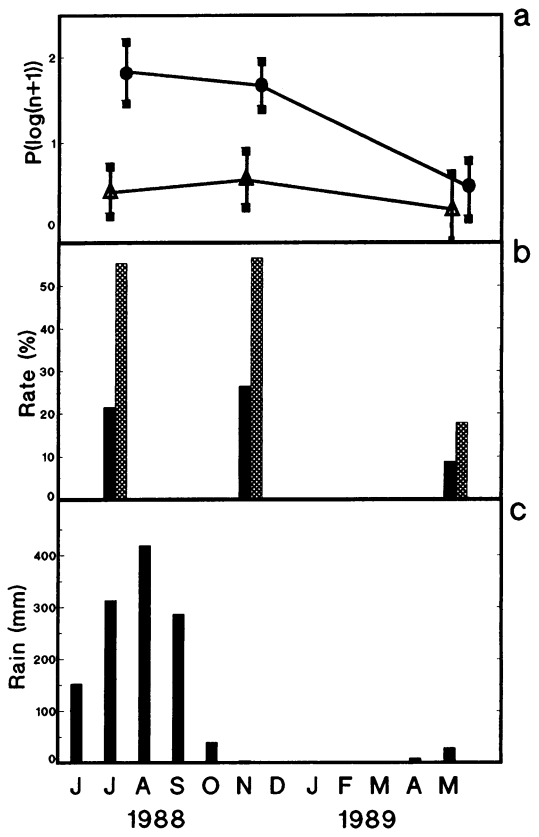

FIG. 1. Annual changes of rainfall and parasitic indices. (a) Parasite density [arithmetic mean of log (n + 1), where n is the number of parasites per microliter, ± SEM] observed for the group of adults (△) and children (●) in Safo. (b) Parasite rate (percent positive thick smears) of each group. Symbols: ▮, adults; ▩, children. (c) Rainfall per month measured in Bamako.

The assay was standardized by using a set of positive sera and 20 serum samples from European patients suffering from diseases unrelated to malaria (5). The cutoff value for considering a serum positive was calculated as the mean of the negative controls + 3 standard deviations (optical density, 0.21 for M6 and 0.23 for M7). No difference between test sera and negative controls was observed when DHFR fused to six histidine residues was used as antigen in the ELISA. Paired test sera were always analyzed on one plate to exclude interexperimental errors.

RESULTS

Malaria in the study population. Blood was taken by fingerprick and intravenously from 37 adults (age, 18 to 73 years; mean, 35.5 years; standard error of the mean [SEM], ±2.29) and 57 children (age, 2 to 9 years; mean, 5.61 years; SEM, ±0.29) from the village of Safo, Mali, on three occasions. The time points were selected with respect to the strong seasonal changes in malaria transmission as a consequence of the seasonal rainfalls in the southern savanna (Fig. 1c).

TABLE 1. gp190 fragments: polymorphism and mapping of epitopes recognized by monoclonal antibodies[a]

Fragment	AA position	Homology	Monoclonal antibody
MAD20 derived			
M4[b]	304–343	P	12.1
M6	384–595	D	
M7	595–897	D	
M8	898–1079	D	
M9[b]	1078–1251	C and D	9.2
M10	1250–1398	D	
M11	1397–1563	D	
K1 derived			
F2	106–321	C and P	
F5	671–833	D	
F7	915–1100	C and D	
F8	1038–1224	C and D	
F10	1412–1608	C and D	6.1, 13.1, 17.1, 2.2, 7.5, 12.8
F10-*RsaI*[b]	1412–1491	D	6.1, 13.1, 17.1
F10-*DraI*[b]	1465–1608	C	2.2, 7.5, 12.8

[a] The amino acid (AA) positions of the K1-derived gp190 fragments are given (10a). The positions of the MAD20 fragments and the classifications of the sequences into conserved (C) and dimorphic (D) are as proposed by Tanabe et al. (19). Polymorphic sequences (P) have been shown not to follow the dimorphic rule (2, 8, 17, 19). For details on the monoclonal antibodies see references 12 and 13. Epitopes recognized by antibodies 2.2, 7.5, and 12.8 were sensitive to β-mercaptoethanol.

[b] Fragments not tested with the sera in this study.

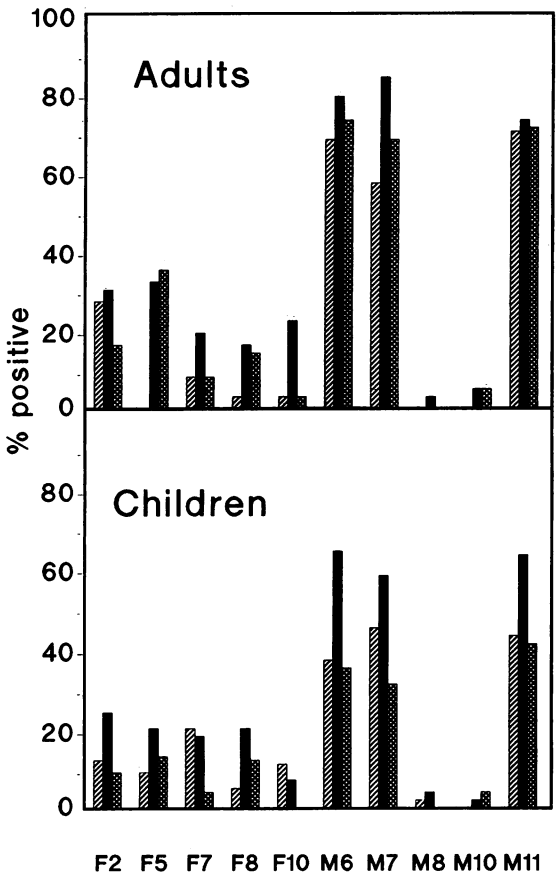

FIG. 2. Seasonal changes in percentage of positive reactions observed for the sera in immunoblots with the gp190 fragments. Sera were taken during July 1988 (▨), November 1988 (■), and May 1989 (▩).

In October 1988, at a time when transmission was most likely at its peak, the mean inoculation rates were calculated to be 0.52 infective bite per night when mosquitoes were caught by insecticide spraying and 2.01 bites per night when insects were collected by hand. At the end of the dry season (May 1989), no infected mosquito was detected.

Parasite rate and parasite density were lower in the adult population than in children (Fig. 1a and b). The differences were statistically significant in July and November ($P < 0.01$; χ^2 test for parasite rate and Student's t test for parasite density; for all statistical tests, see reference 1), but not in May ($P > 0.05$). Within each group the distribution of the parasite load did not differ between July and November, while a significant decrease was observed from November to May (Fig. 1a; $P < 0.01$, paired t test).

Recognition of gp190 fragments by sera. Recombinant fragments covering most of the gp190 genes from the MAD20 and K1 isolates were expressed in *E. coli* as fusion proteins to DHFR and CAT, respectively (Table 1) (15). The MAD20-derived fragments M6, M7, M8, M10, and M11 were used to analyze antibodies specific for the MAD20-type gp190 as they are different from the corresponding K1 sequence. For monitoring the antibody response to conserved epitopes, fragments F2, F7, F8, and F10 originating from the K1 strain were chosen, because they contain most of the highly conserved regions of the gp190 gene. Fragment F5 was included to detect antibodies specific for the K1-type gp190 as it was shown to be highly immunogenic (15).

Analysis of the frequencies of positive reactions in immunoblots to the set of gp190 fragments showed that both adults and children recognized the three MAD20-derived dimorphic fragments M6, M7, and M11 with highest frequencies. Only a small proportion of the sera reacted with the constant or dimorphic fragments from K1 (Fig. 2).

Polymorphism of gp190 expressed by local parasites. The low frequency of K1-specific antibodies in the sera of the inhabitants of Safo could be attributed to a lower prevalence of the K1-type main allele in the parasite population of this area. This hypothesis was tested by culturing blood from eight highly parasitized children, using the candle jar method (20). After 1 to 2 days, parasites matured from the ring to the schizont stage. The parasites were serotyped by immunofluorescence, using a collection of monoclonal antibodies (9, 10, 12, 13) that could discriminate among the conserved, dimorphic, and polymorphic epitopes (Fig. 3). All schizonts displayed conserved epitopes, thus showing gp190 expression. Furthermore, all parasites were stained by antibodies directed against the dimorphic regions of the MAD20 isolate. By contrast, no isolate reacted with antibodies specific for the dimorphic epitopes of the K1 type, indicating that the K1 allele was not present in the eight tested blood samples.

Several monoclonal antibodies recognized only some isolates or only a proportion of the parasites within one isolate in immunofluorescence. Monoclonal antibodies 12.2 and 3D3 appear to recognize epitopes within a region which contains tripeptide repeats and which is highly polymorphic (10, 11). Antibodies 10.2B, 13.2, 9.5, 12.1, and 80cl8 are most likely directed against a second region which does not follow the dimorphic rule (homology block 4 [17, 19]). This was confirmed for monoclonal antibody 12.1, which recognizes in immunoblot the MAD20-derived fragment M4 containing this part of the molecule (Table 1).

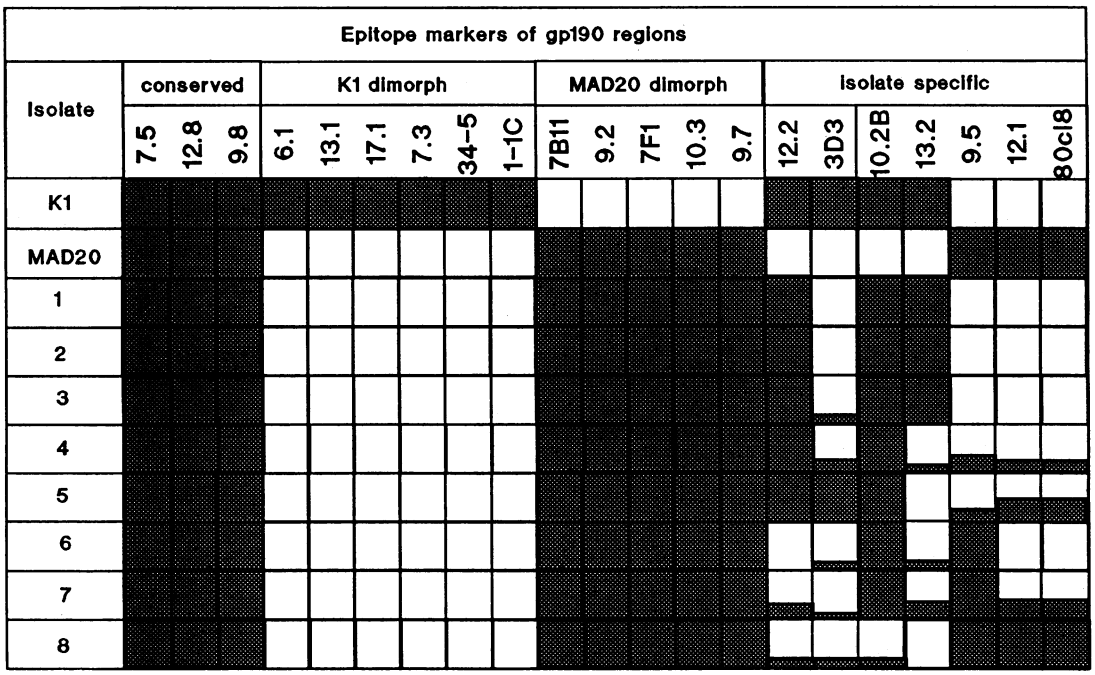

FIG. 3. Reactivity of gp190-specific monoclonal antibodies in immunofluorescence with schizonts cultured from eight individuals from Safo. Isolates K1 from Thailand and MAD20 from Papua New Guinea (13, 19) served as controls. If not all schizonts within an isolate were recognized, the respective frequency of positive parasites is depicted as relative bar height. For location of epitopes recognized by monoclonal antibodies used, see references 8, 9, 10, 12, and 13 and Table 1.

The epitopes of several other monoclonal antibodies used in this study were also successfully mapped to distinct regions of the MAD20- or K1-derived gp190 (Table 1).

Longitudinal analysis of the humoral response to the gp190 fragments in adults and children. Positive reactions of the sera to the gp190 fragments were analyzed with respect to age of the individuals and rate of transmission. Fragments M6, M7, and M11 were recognized by sera from adults at a higher frequency than by sera from children at all three time points (Fig. 2). The differences were statistically significant for all three fragments in May 1989 at the end of the dry season. In July and November 1988, a significant difference was only observed for M11 and M7, respectively ($P < 0.01$; χ^2 test). In the group of children the proportion of positive reactions to these fragments increased during the transmission season, whereas in the group of adults no significant changes in the number of positive signals obtained in immunoblot were observed during the year.

Two of the three immunodominant fragments were examined in ELISA, using the purified proteins His-M6 and His-M7. As observed in the immunoblot assays for both fragments, the proportion of sera positive in the ELISA was always higher for adults than for children. The percentage of sera showing positive reactions with M6 and M7 did not differ significantly between ELISA and immunoblot (data not shown).

The ELISA allowed us to detect quantitative differences in antibody levels within and between the two age groups at different time points during the year (Table 2). In both groups the highest antibody levels were detected at the end of the transmission season. Although the mean optical density was consistently higher for the adults, the significance of the differences observed between adults and children was dependent on the transmission situation. The greatest significance was observed when sera from the end of the dry season were compared; the least was seen with sera

TABLE 2. Comparison of results obtained in ELISA with M6 and M7

Date sera were collected	His-M6			His-M7		
	Mean optical density (±SEM)		Age groups compared[a]	Mean optical density (±SEM)		Age groups compared[a]
	Adults (n = 37)	Children (n = 57)		Adults (n = 37)	Children (n = 57)	
July 1988	0.53 (±0.07)	0.40 (±0.06)	1.55 $P < 0.2$	0.61 (±0.07)	0.46 (±0.06)	1.77 $P < 0.1$
November 1988	0.95 (±0.10)	0.56 (±0.08)	3.43 $P < 0.01$	1.04 (±0.09)	0.67 (±0.07)	3.26 $P < 0.01$
May 1989	0.61 (±0.08)	0.26 (±0.07)	3.95 $P < 0.001$	0.68 (±0.08)	0.35 (±0.06)	3.53 $P < 0.001$
Seasons compared[b]	33.41 $P < 0.001$	35.03 $P < 0.001$		37.35 $P < 0.001$	45.95 $P < 0.001$	

[a] Student's t test (value for t is shown).
[b] Friedman test for July, November, and May (value for χ^2 is shown).

taken at the beginning of the wet season (Table 2). Within each group the M6/M7 specific antibody levels showed highly significant variation during the year.

DISCUSSION

Our longitudinal analysis of naturally acquired antibodies against the gp190 of *P. falciparum* allows conclusions with regard to the strain specificity as well as the temporal development of the humoral immune response in individuals living in a malaria endemic area.

When sera of 94 individuals living in Safo, Mali, were tested for antibody reactivity against conserved and dimorphic parts derived from the gp190 of K1 and MAD20 isolates, respectively, a clear bias for MAD20 specificity was observed. This is in contrast to our earlier findings in Burkina Faso where dimorphic regions of the gp190 of both the MAD20 and K1 isolates were recognized with high frequency (15). We interpret this difference as a consequence of the different frequencies of the two main gp190 alleles within the *P. falciparum* parasites of the two areas. This interpretation was confirmed by analyzing the gp190 antigen of the local parasites of Safo with a set of gp190-specific monoclonal antibodies differing in strain specificity (9, 10, 12, 13). No schizonts were detected which reacted with monoclonal antibodies specific for the dimorphic class of gp190 variants represented by the K1 strain (13, 19). By contrast, all eight isolates tested were positive with antibodies which recognize the dimorphic parts of MAD20. Therefore, we conclude that the parasite populations of the areas investigated in Burkina Faso and Mali differed genetically and that antibodies against particular sequences (F5 of K1 and M6, M7, and M11 of MAD20) are indicative of the presence of the respective dimorphic allele within the infecting parasites. This analysis clearly demonstrates that antibodies acquired upon natural infections with *P. falciparum* are specifically directed against the antigenic variants of gp190 which were present during the infection.

Polymorphism within the gp190 antigen of the parasite isolates of Safo was observed for epitopes located in two parts of the molecule which are known not to follow the dimorphic rule (homology blocks 2 and 4 [2, 8, 17, 19]). Therefore, the variability of gp190 in the parasite population in Safo appears to be restricted to subserotypes of the MAD20-type allele.

Qualitative and quantitative analyses of the sera in immunoblots and ELISA revealed seasonal differences within each age group as well as age-related differences between the groups.

A correlation between the intensity of transmission and gp190-specific antibody levels in the population was observed, whereby the highest levels were found towards the end of the malaria transmission season (November). At the end of the dry season (May), after a period of low transmission, both the rate of positive reactions in immunoblots and the mean ELISA values with M6 and M7 were much lower. This indicates that a drop in transmission is accompanied by a drop in antibody levels against blood stage antigens and confirms data obtained previously (15, 16).

When seasonal changes in the antibody levels against the dimorphic regions of gp190 were compared between adults and children, an interesting feature emerged. At the end of the dry season both the antibody levels and the proportions of individuals reacting to M6 and M7 were significantly lower in the group of children. This occurred despite the fact that a higher proportion of children was infected and therefore exposed to a higher antigenic stimulus during the entire transmission season. Furthermore, a higher proportion of children remained parasitized during the dry season. Since during the transmission season the proportion of positive reactions as well as the levels of specific antibodies did not differ to the same extent between adults and children, the differences observed at the end of the dry season indicate that the gp190-specific humoral response in children is shorter-lived than that in adults. This suggests an age-related qualitative difference in the dynamics of the immune response against blood stage antigens of *P. falciparum*. The low levels of antibodies found in children before the beginning of a new transmission season may explain the high risk of reinfection in this group.

In a malaria endemic area, like the one described in this study, *P. falciparum* infections occur primarily when transmission is high. Subsequently, clearing of the parasites is observed (Fig. 1). This may be explained by the short-lived antibody response to the local set of polymorphic antigens: the specific immune response induced by a particular variant may be able to clear this infection but may not protect against superinfection by another antigenic type. However, as the response to the first variant declines rapidly, reinfection by these parasites again becomes possible. Therefore, a limited antigenic polymorphism, as indeed found so far for gp190, would be sufficient for repeated infections in hosts mounting a protective but short-lived immune response. A subunit vaccine based on such antigens should consequently be able to induce a longer-lasting immune response to a broader spectrum of variants than naturally observed.

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