Antibodies to the Conserved C-Terminal Domain of the *Plasmodium falciparum* Merozoite Surface Protein 1 and to the Merozoite Extract and Their Relationship with In Vitro Inhibitory Antibodies and Protection against Clinical Malaria in a Senegalese Village

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Antibodies to *Plasmodium falciparum* C-terminal merozoite surface protein 1 (PfMSP-1p19) have been correlated with protection against malaria, but this association may apply to many merozoite antigens. To address this question, we conducted a prospective serological study of 205 individuals in an active 5-month clinical survey in a Senegalese village where malaria is mesoendemic. Before the 2000 rainy season, antibody responses specific for recombinant baculovirus PfMSP-1p19 or merozoite extracts were compared with 2 in vitro functional antibody activities (inhibition of parasite grown and erythrocyte invasion) and with the number of clinical episodes during 5 months of follow-up. Antibody levels to PfMSP-1p19 and merozoite extract correlated, respectively, with erythrocyte invasion and parasite growth inhibition. Although antibody levels to both antigen preparations were associated with age, functional parameters were not. High levels of anti–PfMSP-1p19 immunoglobulin G were associated with reduced malaria in an age-adjusted multivariate analysis. These results support baculovirus PfMSP-1p19–based vaccine development.

Progress toward the development of a malaria vaccine requires a better understanding of the immune mechanisms that contribute to the natural protection of individuals living in endemic areas. Seroepidemiologic studies have found associations between antibody responses to specific *Plasmodium falciparum* antigens and protection against clinical disease. Several antibody-mediated mechanisms operate to restrict the multiplication of parasites, including the inhibition of erythrocyte invasion [1, 2], the inhibition of merozoite release [3], intracelluar parasite killing [4], and the destruction of infected red blood cells (RBCs) [5, 6]. In the context of vaccine development, antigens that show restricted diversity are of particular interest. Of these, one of the most conserved antigens is the C terminal of merozoite surface protein (MSP)–1 [7, 8]. MSP-1 is produced as a high-molecular-mass precursor that is membrane anchored via a glycosyl phosphatidyl inositol moiety. It undergoes 2 successive proteolytic processing steps during merozoite maturation [9], leading to a final conserved C-terminal membrane-anchored moiety called “PfMSP-1p19,” which consists of 2 structurally constrained epidermal growth factor (EGF)–like domains [10, 11]. The response to PfMSP-1p19 is complex and includes antibodies that inhibit erythrocyte invasion by...
merozoites, as well as those that compete with the inhibitory antibodies [12, 13]. The inhibition of erythrocyte invasion has been associated with the presence of antibodies blocking the final proteolytic step that produces PfMSP-1p19 [13, 14] and with protection in a murine model that used PfMSP-1p19 transgenic Plasmodium berghei parasites [15].

The antibody response to PfMSP-1p19 has been positively correlated with clinical immunity in children and adults in some settings [16–21] but not others [22, 23]. The relevance of these associations has rarely been evaluated with regard to their putative underlying function—namely, the inhibition of merozoite invasion. Recently, O’Donnell et al. [24] reported that antibodies to PfMSP-1p19 are major contributors of the invasion inhibitory activity present in the serum of immune adults. However, it is not clear how this relates to protection against disease or to other potentially protective mechanisms that target other merozoite antigens. The aim of the present study was to document the relationship of the specific anti–PfMSP-1p19 antibody response to the more general antimerozoite antibody response and to investigate their association both with growth and/or invasion inhibition in vitro and with protection against clinical malaria in an endemic population. We conducted a prospective serological study in Ndiop, a Senegalese village with moderate and seasonal transmission [25], where a longitudinal prospective study has been ongoing for >10 years [26, 27]. The study included 205 plasma samples collected just before the rainy season, followed by the monitoring by active daily case surveillance of episodes of malaria throughout the subsequent transmission season. The antimerozoite antibodies were evaluated by use of a crude merozoite extract, prepared from the FCR3 reference strain. Importantly, a baculovirus-expressed and -secreted recombinant protein was used to evaluate the anti–PfMSP-1p19 antibody response, because it involves reduction-sensitive epitopes [2, 28, 29]. This system has been shown to ensure a homogeneous folding of the structurally constrained recombinant product, whose crystal structure revealed proper folding of both EGF domains [11]. In addition, we evaluated individual serum samples with regard to 2 in vitro functional assays—inhibition of parasite growth and of erythrocyte invasion—and their relationship with the number of clinical episodes during the subsequent transmission season.

SUBJECTS, MATERIALS, AND METHODS

Study area, design, and procedures. The study was conducted in Ndiop, Senegal, a village with moderate seasonal transmission [25, 26], after approval was obtained from the ad hoc Ethics Committee of the Ministry of Health. The follow-up protocol was explained to the assembled village population, and informed consent was obtained from the villagers. Any individual could withdraw from the study and the follow-up procedure at any time.

Briefly, in July 2000, 205 healthy villagers (108 male and 97 female; age range, 3.6–75 years; mean age, 23.9 years) were enrolled for a cross-sectional prospective follow-up. The cohort was composed of 36, 4, and 165 individuals with hemoglobin type AS, AC, and AA, respectively. Samples were obtained from all villagers between 17 July and 1 August 2000. Samples from 37 villagers showed microscopically positive peripheral parasitemia at the time of blood sampling (range, 0.5–80 trophozoites/100 leukocytes). The detection threshold of the microscopic examination is 1 parasite/μL in our laboratory [30]. None of the villagers recruited had used antimalarials for at least 4 weeks before blood was drawn. After venous puncture, plasma and RBCs were separated by centrifugation and stored at −20°C.

Active clinical surveillance was done over a 5-month period encompassing the malaria transmission season, from 1 August to 31 December 2000, as described elsewhere [26, 30, 31]. The protocol included the notification of all febrile episodes and of the controlled use of antimalarial drugs by the medical staff. Each village was visited daily at home for clinical surveillance; blood films were made in case of fever and were read extemporaneously. A malaria attack was operationally defined as an association of symptoms suggesting malaria with parasitemia levels >30 trophozoites/100 leukocytes. Antimalarial drug treatment was administered by the medical staff after each positive diagnosis for malaria. A total of 278 clinical episodes were treated with antimalarials. Blood films were subsequently made a second time for the proper quantification of parasitemia.

In parallel, the entomological inoculation rate (EIR) was monitored weekly, as described elsewhere [25]. The cumulative EIR for the entire transmission season was estimated to be 50.75 infective bites/individual between the end of July and mid-October 2000. No transmission was recorded in November and December 2000 in the village.

Antigens and ELISA procedure. The merozoite extract was prepared from synchronous FCR3 parasites cultivated on O+ erythrocytes and 10% human serum in candle jars, as described elsewhere [31]. Merozoites, collected after stepwise centrifugation at 2000 and 4000 g, were washed 3 times in sterile PBS, counted, and frozen.

The extract was used to coat MaxiSorp plates (Nunc) at 10 μg/mL [32]. MSP-1p19 (Palo Alto allele) was produced in Spodoptera frugiperda (Sf9) or Trichoplusia ni (High Five; Invitrogen) insect cells infected with the recombinant baculovirus, purified by metalloaffinity chromatography [33], and used to coat Immulon-4 plates at a concentration of 0.5 μg/mL, after dilution in sterile PBS.

ELISAs for the determination of IgG responses were performed, as described elsewhere [31, 32, 34]. Plasma samples were tested in duplicate at a dilution of 1:200. A negative control (a pool of European and/or African nonimmune serum
samples) and a positive control (a pool of 25 serum samples from clinically immune adults living in Dielmo and Ndiop) were included in each assay, to ensure comparability between the plates. Results were expressed as optical-density ratios (optical density of the sample:optical density of the naive serum pool) [31, 34]. Serum samples from individuals with an OD ratio ≥2, which exceeds the signal of naive control samples ± 2 SD (OD ratio, 1.9), were considered to be seropositive.

Growth-inhibition assay (GIA) and erythrocyte invasion inhibition assay (EIIA). The GIA was done by use of sorbitol-synchronized cultures. Young trophozoite-stage parasites were adjusted to 0.4% parasitemia at 1.5% hematocrit in a 96-well tray. Plasma, tested in triplicate at a 1:10 dilution in RPMI 1640 and 0.5% Albumax (Gibco), were incubated for 24 h at 37°C in candle jars. Medium was removed, and 25 μL of [3H] hypoxanthine was added (1 μCi/well); then, samples were further incubated for 24 h. Plates were then frozen and thawed to lyse infected RBCs. Samples were transferred to glass-fiber filters and quantified by use of a β-scintillation counter (Trilux; Wallac). The EIIA was done essentially as described by O’Donnell et al. [24], except that the FCR3 strain was used [35].

Randomly chosen serum samples (n = 94) from sex- and age-stratified groups (0–14, 15–29, and ≥30 years) were tested in parallel in both assays. This included 51 males and 43 females; the mean age was 25.7 years (range, 3.6–75 years). Negative control samples consisted of medium alone, a commercial pool of samples from nonimmune European donors (Valbiotech), and a pool of 20 African nonimmune serum samples. A pool of 25 immune serum samples was used as a positive control [32, 35]. The percentage of inhibition was calculated as ([mean cpmnegative control − mean cpm sample]/mean cpmnegative control) × 100.

Statistical analysis. Comparisons of antibody levels and/or the inhibition of growth or invasion in different groups were done by means of the Wilcoxon signed rank test and the Spearman rank correlation test for nonnormally distributed paired data. P < 0.05 was considered to be significant.

A Poisson regression model was used to analyze the relationship between the antibody response and the incidence of malaria during the follow-up period. For the analysis, an attack of P. falciparum malaria was defined as the presence of fever or symptoms suggesting malaria that were associated with >30 P. falciparum trophozoites/100 leukocytes, as ascertained by the reexamination of all slides by a highly experienced microscopist. This showed that 31 of 278 antimalarial treatments administered did not fulfill the strict definition of an attack of P. falciparum malaria. There were 2 episodes of P. malariae infection, 6 episodes of P. ovale infection, and 23 episodes with samples showing <30 P. falciparum trophozoites/100 leukocytes. For each villager, the follow-up time was calculated as the number of days actually spent in the village during the 5 months of follow-up. Seven villagers, who were out of the village for >30 days during the follow-up period, were excluded from the malaria incidence analysis. Thus, the analysis included 192 individuals, who had a total of 278 episodes of clinical malaria during the follow-up period. The incidence of clinical malaria (per 1000 person-days) was 29 (range, 2–35) for the age group 0–14 years (n = 77), 9 (range, 6–13) for 15–29 years (n = 58), and 2 (range, 1–4) for ≥30 years (n = 57) (P < .001, Wald test). Attacks of malaria were considered to be independent if they were separated by >15 days. The follow-up time was adjusted for individuals who had attacks of malaria by excluding from the days at risk a period of 15 days after the diagnosis of malaria. It was also adjusted for the individuals who received an antimalarial treatment without fulfilling the strict definition of malaria by excluding a period of 8–15 days after the first day of treatment (8, 10, and 15 days for quinine, chloroquine, and sulfadoxine-pyrimethamine, respectively).

The optimal age stratification was based on the age distribution of the parasitological and clinical data available for this setting, based on the 10-year longitudinal follow-up data for the entire population [26, 30]. The ages of 15 and 30 years were used as cutoffs [31]. First-level interactions between variables were checked and were included in the model when they were significant. The antibody level stratification was determined by use of Akaike’s information criterion. Statistical analyses were performed with Egret (version 3.01; Cytel) and Statview (version 5.0; SAS Institute) software.

RESULTS

Prevalence and quantification of antibodies to the merozoite extract and to PfMSP-1p19. The prevalence of serum samples reacting with the merozoite extract and the PfMSP-1p19 antigen was very high—96% and 79% of villagers scored as positive, respectively. The mean optical-density ratios to the merozoite extract and PfMSP-1p19 were 3.1 ± 0.6 (OD ± SD, 1.1 ± 0.33) and 8.1 ± 6.1 (OD ± SD, 0.97 ± 0.79), respectively. For both responses, the mean and median optical-density ratios coincided. The IgG responses to the merozoite extract and to PfMSP-1p19 were positively correlated (r = 0.36; P < 10−4), confirming previous results [31], and both were positively associated with age (r = 0.32 and 0.37 for the merozoite extract and MSP-1p19, respectively; P < 10−4) (figure 1). They were not associated with sex, hemoglobin type, or detectable parasitemia level on the day of blood sampling.

Inhibitory activity in functional assays and its relationship with ELISA responses. A subset of 94 plasma samples from subjects with a representative age distribution was analyzed for the presence of antibodies affecting the multiplication of parasites by use of 2 functional assays, a GIA and an EIIA. Interestingly, for both assays, the antibody response showed a distribution close to normal (P = .1 for both, Shapiro-Wilk) (figure 2A and 2B.
for GIA and EIIA, respectively). A large proportion of individuals had inhibitory antibodies (i.e., showed >10% inhibition in the assay). All plasma samples presented inhibitory activity in the GIA, showing 26%–81% inhibition. The mean value for GIA was quite high, 55.7% ± 13%. For the EIIA, only 2 of 94 individuals displayed >10% inhibition. The mean value for EIIA was 39.5% ± 15.2% (range, 10%–75%).

The inhibitory responses in the GIA and EIIA were positively correlated (rho = 0.42; P < 10^-4), but, importantly for both assays, there was no association of inhibitory activity with age. Interestingly, the ELISA response to the merozoite extract was positively correlated with inhibitory activity in the GIA (rho = 0.30; P = 0.004, Spearman rank test) but not in the EIIA. A dual-level stratification of the antibody response to the merozoite extract showed a capacity for higher growth inhibition in the GIA (P = 0.02) for individuals with IgG ratios ≥2.5 (figure 3A).

The unstratified antibody response to PfMSP-1p19 showed no correlation with inhibitory activity in the GIA or the EIIA. However, a dual-level stratification showed that samples with a high level of anti-PfMSP-1p19 antibody (i.e., OD ratio, ≥7) had significantly higher inhibitory activity in the EIIA (figure 3B; P = 0.01) but not in the GIA (figure 3A). Thus, the ELISA responses to the merozoite extract and to PfMSP-1p19 were associated with distinct in vitro functional assays.

**Relationship between antibody responses and the number of clinical episodes.** The relationship between the dichotomized antibody response and the incidence of clinical malaria episodes during the 5-month follow-up period was analyzed by use of an age-adjusted Poisson regression model. The inhibitory activity in the GIA or EIIA was unrelated to protection against malaria during the subsequent 5 months. Likewise, the unstratified and stratified IgG response to the merozoite extract was not significantly associated with the incidence of malaria (P = 0.97). In contrast, there was a significant association of the stratified anti–PfMSP-1p19 response (OD ratio, ≥7 vs. <7) with a reduced incidence of episodes of malaria (incidence rate ratio [IRR], 0.77; range, 0.60–0.99; P = 0.047).

A multivariate analysis showed that the variables significantly associated with the incidence of clinical malaria were (1) age (15–29 vs. ≥30 years, IRR, 4.0; <15 vs. ≥30 years, IRR, 12.7; P < 0.001 for both), (2) hemoglobin characteristics (AS vs. others, IRR, 0.55; P = 0.001), (3) positive parasitemia at enrollment (IRR, 0.69; P = 0.02), and (4) the IgG response to PfMSP1-1p19 (OD ratio, ≥7 vs. <7; IRR, 0.73; range, 0.55–0.96; P = 0.03).

A striking characteristic of the relationship of the anti–PfMSP-1p19 IgG response with protection is illustrated in figure 4. The cumulative incidence of episodes of malaria was quite distinct in the 3 age groups considered. The incidence of malaria in the ≥30-year age group was low, whatever the level of anti–PfMSP1-1p19 IgG; it was 2–3 fold higher in the 15–29-year age group and was also essentially unrelated to the anti–PfMSP-1p19 response. However, there was a marked dichotomy in the younger age...
group, which accounted for 73% of the clinical attacks recorded during the 5-month follow-up period. The children with a relatively lower level of anti–PfMSP-1p19 IgG had a much higher incidence of malaria than those of the same age with relatively higher antibody levels of PfMSP-1p19. This dichotomy was particularly marked during the first month of the survey but remained throughout the 5-month period.

DISCUSSION

A high percentage of the serum samples from the endemic population that we tested were reactive with the merozoite extract and/or baculovirus recombinant PfMSP-1p19, by both ELISA and the functional assays. Because no infected mosquito was captured in Ndiop from the end of September 1999 through the end July 2000, this denotes the persistence of substantial levels of specific (or cross-reacting) antibodies over 10 months of highly reduced or interrupted transmission. It is unclear whether this reflects continuous antigenic stimulation resulting from chronic, low-density parasite carriage or slow decay of strong responses triggered or boosted during the transmission season. Short-lived antibody responses to PfMSP-1 have been reported in humans [1, 22].

The magnitude of antibody responses relating to the 4 parameters that we investigated is remarkable in view of the inherent limitation of the tests due to parasite polymorphism. Previous molecular epidemiology studies conducted in this setting have indeed indicated that allelic diversity is quite large [36–39]. Because the merozoite extract derives from the FCR3 line, which was also used for the inhibition assays, only a single allelic type for the various merozoite polymorphic antigens—such as MSP1–4 or AMA1—was tested. Thus, the merozoite extract ELISA and the inhibition assays measured responses to conserved antigens and to an unknown fraction of the overall variable antigenic repertoire of the local parasite population. Allelic polymorphism is less of an issue for PfMSP-1p19, which is highly conserved. Indeed, PfMSP-1p19 sequences derived from 50 of 52 isolates collected in Ndiop and in the nearby village of Dielmo corresponded to 2 alleles differing at a single dimorphic position (E-KNG and Q-KNG), the former of which corresponds to the Palo Alto allele used in the present study (S. Rosario and A. Manampieri, unpublished data). Previous work has indicated that, as expected with an antigen as conserved as PfMSP1-p19, there is substantial cross-reactivity between allelic forms by antibodies from individuals exposed to malaria [28].

Antibodies to PfMSP-1p19 and to the merozoite extract were positively correlated, and both antibody responses were age related, confirming previous observations made in this setting 3 years earlier, also just before the onset of the malaria transmission season [31]. An age-related IgG response to PfMSP-1p19 has been documented in many endemic areas [18, 20, 21, 28, 40]. Depending on the study site, the prevalence of antibodies varies from 28% to 77% [20, 21, 23, 28, 40, 41]. This cannot be attributed to variable intensity of transmission and probably reflects suboptimal evaluation of the serological response resulting from the use of recombinant antigens produced in expression systems that do not ensure the homogeneous folding of conformation-constrained epitopes provided by the baculovirus expression system [2, 33, 42].

Interestingly, neither growth nor invasion inhibitory activities were age related, although serum samples from all villagers showed some growth inhibitory activity, and a very large proportion of the population had invasion inhibitory antibodies. Because the use of antimalarials in the village is strictly monitored, invasion or growth inhibition due to the presence of these drugs in the plasma can be excluded. Thus, the inhibition activities can be attributed to specific antibodies, and this interpretation is supported by their correlation with specific ELISA responses. Growth inhibition was associated with the presence of antibodies reacting with the merozoite extract, whereas the inhibition of erythrocyte invasion was related only to high levels of PfMSP-1p19–specific antibodies. This suggests that the GIA and EIIA measure independent responses that
probably involve distinct antigen/antibody interactions. The association of anti–PfMSP-1p19 antibodies with the invasion inhibitory response is consistent with the correlation of higher levels of anti–PfMSP-1p19 IgG1 antibody with lower parasite density in Kenyan children [19] and with the inhibitory activity of serum samples from malaria-exposed immune adults on PfMSP-1p19–transgenic lines [24]. We show here that this activity is not dependent on age and is observed only in individuals who have high levels of antibody specific for PfMSP-1p19. Thus, it is not necessarily restricted to “immune adults.” The association of invasion inhibitory activity with the response to PfMSP-1p19, rather than with the response to the merozoite extract, is consistent with competing “blocking antibodies” reacting with PfMSP-1 sequences outside of PfMSP-1p19 [13]. An alternative explanation is that the response to PfMSP-1p19 is masked by responses to other merozoite antigens within the merozoite extract. Further work is needed to explore these possibilities.

Protection against clinical malaria was associated with a single specific immune signature—namely, the presence of high levels of antibody to PfMSP-1p19. In a similar prospective study conducted in Ndiop in 1997, a reduced incidence of clinical malaria was associated with the response to the merozoite extract (IRR, 0.804), but the association with the response to PfMSP-1p19 did not reach statistical significance because of an age-related confounding effect [31]. We attribute this discrepancy to the larger sample size of the present study (205 vs. 110 subjects in the 1997 study). The data in the present study confirm the association of high levels of anti–PfMSP-1p19 IgG with protection against clinical malaria in Gambian children [21]. Interestingly, in Gambian children, as in the Ndiop villagers, there was no association with the frequency of responders to PfMSP-1p19, consistent with observations in other settings [19, 21, 22].

The association of protection against clinical malaria with high anti–PfMSP-1p19 IgG levels, but not invasion inhibitory antibodies, is in apparent conflict with the conclusions drawn from a newly developed mouse model of infection with transgenic P. chabaudi parasites expressing PfMSP-1p19 [15]. In mice immunized by infection and cure with transgenic parasites, protection following subsequent blood-stage challenge was not associated with levels of IgG to PfMSP-1p19 but was correlated with the levels of antibody inhibiting P. falciparum erythrocyte invasion in vitro [15]. However, “protection” in this mouse model refers to the prevention of lethal hyperparasitemia, with reduced peak parasitemias and reduced clearance time. This differs substantially from the definition of protection against clinical malaria in humans. Indeed, the peak parasitemia observed in the “protected” mice (14.8%) would qualify the infection as hyperparasitemia in human malaria, where “protection” refers to the control of peripheral parasitemia to <0.02% in the absence of clinical symptoms. In addition, homologous challenge is very unlikely in a mesoendemic setting such as...
Acknowledgments

We thank all the villagers from Ndiop who participated in the study and O. Garraud (University St. Etienne, France) for helpful discussions.

References


Ndiop. Thus, the mechanisms implicated in protection in the mouse model and in endemic areas may not totally overlap.

The present prospective survey allowed us to investigate correlations of specific serological responses with protection against clinical malaria during the subsequent transmission season. The observed correlation of protection with antibody levels at recruitment obviously does not preclude the contribution of additional immune responses to protection, including the rapid boosting, on parasite exposure, of antimerozoite responses that were subliminal at the onset of transmission or the mounting of novel responses directed against the variant RBC surface antigens [43]. The age-independent, hemoglobin type-independent, association of high anti–PfMSP-1p19 IgG levels with a reduced incidence of clinical episodes argues strongly in favor of further vaccine development based on this antigen. Our data are consistent with the interpretation that high levels of antibodies to PfMSP-1p19 contribute to clinical protection, possibly by inhibiting the merozoite invasion of erythrocytes. Because conformation-dependent antibodies to PfMSP-1p19 are mainly cytophilic [18, 34], they could also be involved in other immune mechanisms, such as monocyte-mediated, antibody-dependent parasite killing [4].


