MAJOR ARTICLE

Distinct Surrogate Markers for Protection against *Plasmodium falciparum* Infection and Clinical Malaria Identified in a Senegalese Community after Radical Drug Cure

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Plasmodium falciparum **expresses many antigens, which elicit various immune responses in exposed individuals, but no simple surrogate marker for protection has yet been developed. In this prospective survey, we looked for immune responses predictive of protection at various stages of progression from parasite inoculation to onset of disease. We studied 110 Senegalese volunteers from an area in which malaria is mesoendemic after they had received eradication therapy. We evaluated 4 protection-related outcomes (reappearance of parasitemia, duration of asymptomatic carriage, time to first clinical episode, and incidence of clinical episodes) in terms of levels of immunoglobulin G (IgG) against 3 crude parasite extracts and 5 conserved antigens during a 5-month period. Kaplan-Meier estimates and age-adjusted regression models showed these 4 outcomes to be associated with different patterns of IgG response to PfEMP3-cl5 (derived from *P. falciparum* erythrocyte membrane protein 3), PfEB200, MSP–1_{19} (derived from merozoite surface protein–1), $[NANP]_{10}$, infected red blood cell membrane, and merozoite and schizont extracts. It should, therefore, be possible to develop surrogate markers for each end point on the basis of IgG response to a limited number of conserved antigens.**

Individuals living in areas of stable *Plasmodium falciparum* transmission develop immunity to malaria over a period of years, progressively reducing the risk of severe and mild malaria but without completely eliminating the risk of infection. Asymptomatic carriage of *P. falciparum* parasites is therefore frequent. In most areas in which malaria is endemic, the development of efficient protection against mild malaria seems to require numerous infections. This may reflect a need to gradually acquire a large antibody repertoire directed against diverse surface antigens, limited immunogenicity of the conserved antigens, or both. A variety of immune responses directed against numerous parasite antigens have been identified [1–10], some of which are associated with parasite killing in vitro [11–13]. However, we still lack surrogate markers of protection for routine use in the analysis of acquired protection, vaccine development, and assessment of the long-term effect of control measures.

There are several reasons for this lack of surrogate markers. Results from cross-sectional studies that compare children with malaria with asymptomatic children or that compare susceptible children with protected adults are difficult to interpret. Parasite infestation is difficult to distinguish from the clinical disease, malaria, and a further confounding factor is that parasite densities, and hence antigenic loads, differ considerably between the groups compared. Prospective studies involving the longitudinal monitoring of subjects in areas in which malaria is endemic provide a means of gaining

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useful information on the immune status of these subjects. However, the term "protection," as used in investigations of predictive associations, may refer to different aspects of progression, from inoculation with parasite to onset of disease. Some studies have investigated the association between cellular or humoral responses and resistance to reinfection [6, 7, 14], whereas others have investigated the association between immune responses and lower morbidity [1, 5, 8, 15]. Recent studies have reported a significant association between humoral responses to polymorphic surface antigens—such as merozoite surface antigen 1 block 2 [4] (authors' unpublished data) or the clonally variant infected erythrocyte surface adhesin *P. falciparum* erythrocyte membrane protein (PfEMP) 1 [2, 3, 9]—and protection against clinical malaria in African children. The large field diversity of these antigens complicates their use as surrogate markers of protection. There is a need to develop a convenient, strain-independent, surrogate assay for measuring protection against *P. falciparum*. We performed this study with the aim of developing such a test.

We investigated the association between, on the one hand, humoral response to a panel of conserved antigens and crude parasite extracts prepared from a reference strain and, on the other hand, protection acquired by individuals exposed to malaria. To make it possible to associate immune responses with well-defined infection end points, we used individual recordings of the reappearance of parasitemia and occurrence of clinical episodes, which were collected during active longitudinal, parasitological, and clinical monitoring, after eradication therapy, in 110 Senegalese volunteers during a 5-month transmission season (A. Tall, unpublished data; L. Marrama, unpublished data). The present study was conducted in Ndiop, Senegal, a village in which malaria is mesoendemic, transmission is moderate and seasonal [16], and the age distribution of clinical malaria is well documented [17, 18]. We analyzed pretreatment antibody responses to crude schizont, merozoite, and infected erythrocyte membrane (infected red blood cell [IRBC] membrane) extracts prepared from the FCR3 reference strain [19] and to 5 individual conserved antigens that, in other studies, have been reported to be associated with protection. These antigens are encoded by single-copy genes and display no structural similarity, and the antibodies they induce do not cross-react. $[NANP]_{10}$ is derived from the central repetitive domain of the circumsporozoite protein (CS), a major sporozoite surface antigen. Immune response to CS repeats has been associated with protection in areas in which malaria is endemic [10], and the CS-based RTS-S vaccine induces significant protection against reinfection in human subjects [20]. Merozoite surface protein (MSP)–1_{19} is derived from the C-terminal domain of *P. falciparum* MSP-1. Antibodies directed against MSP-1_{19} have been associated with protection against clinical malaria in some African children [5, 21] and account for a large part of the in vitro invasion-inhibitory response [13]. MSP-1_{19} induces sterile immunity in murine or monkey models [22–24]. Thus, both CS protein and MSP-1_{19} are currently prime vaccine candidates. PfEMP3-cl5 is derived from the C-terminal repetitive domain of PfEMP3, a conserved accessory molecule implicated in trafficking the variant surface PfEMP1 adhesin to the erythrocyte membrane [25, 26]. PfEMP3-cl5 is a target of the variant-specific response in the *Saimiri* monkey [28, 29]. The protein is also exposed on the sporozoite surface [27]. Its N-terminal domain induces antibody that inhibits sporozoite invasion [27]. PfEB200 and R23 are derived from IRBC-associated conserved antigens encoded by *Pf332* [30] and *R45* [31], respectively. Both interfere with the opsonization of IRBCs by hyperimmune serum in vitro [11] and induce protective immunity against lethal infection in monkeys [32–34]. The seroprevalence of antibodies directed against PfEMP3-cl5, PfEB200, and R23 has been shown to be associated with age at 2 sites in Senegal, including Ndiop, where this study was performed [35].

We analyzed the prevalence of IgG and levels of antibody directed against these antigens, before eradication therapy, and examined their association with 4 protection-related end points defined for each enrolled individual: time to reappearance of parasitemia (called "reinfection" in some studies [7, 14]), duration of asymptomatic carriage, time to first clinical episode, and incidence of clinical episodes experienced during follow-up.

SUBJECTS, MATERIALS, AND METHODS

Study area, study design, and procedures. The present study was conducted in the village of Ndiop, in an area of moderate, seasonal transmission [16, 17]. This study was approved by the ad hoc Ethics Committee of the Ministry of Health. The protocol was explained to the assembled village population, and informed consent was obtained individually from each participant or his/her parents. Each year, the longitudinal project is reexamined by the Conseil de Perfectionnement of the Institut Pasteur de Dakar and by the assembled village population. Informed consent is individually renewed, and any subject can exclude him/herself from the survey at any time. The study design and procedures were similar to those used in a post–radical cure study conducted in the nearby village of Dielmo [36] and will be reported elsewhere (A. Tall, unpublished data; L. Marrama, unpublished data). In brief, during the period of 4–10 August 1997, 110 villagers (46 male and 64 female), 2.6–73 years old (mean age, 22.1 years), were enrolled for longitudinal prospective follow-up. Individuals with fever (temperature >38°C) and individuals who had received antimalarial treatment on the day of enrollment were excluded. The study ended on 31 December 1997. A capillary blood sample was obtained at enrollment. Plasma and RBCs were separated by

centrifugation and were stored at −20°C. Enrolled subjects were treated with quinine (25 mg/kg/day, divided into 3 equal oral doses administered every 8 h over 7 days; Quinimax; Sonofi), under supervision by the medical field team, to eradicate current malaria infections. We tracked the reappearance of blood forms (reinfection) by obtaining thick blood smears weekly during the first 11 weeks after enrollment. Daily clinical surveillance was performed during the 5 months of follow-up, as described elsewhere [17, 36]. Each subject was visited at home for clinical surveillance, and additional blood films were made in cases of fever. The protocol included the notification of the medical staff of all febrile episodes and the controlled use of antimalarial drugs. A malaria episode was defined as an association of symptoms suggesting malaria with a level of parasitemia of >30 trophozoites/100 leukocytes. An antimalarial drug cure was administered by the medical staff in all cases of malaria episodes. The volunteers complied with the protocol and did not take additional antimalarial medication during follow-up.

The thick blood smears were collected and processed as described elsewhere [17]. We were able to detect ~1 parasite/μL by microscopy.

In parallel, the entomological inoculation rate (EIR) was monitored twice a week by conducting 16 indoor and outdoor human bait catches/night, every Monday and Thursday during the entire study period. The cumulative weekly EIR was estimated from the daily EIR, as described elsewhere [16]. The cumulative risk of receiving 1 infective bite (r) was calculated according to the formula of Krafsur and Armstrong [37]: $r = 1 - e^{-EIR(t)}$, where "t" stands for time. The cumulative risk reached 100% after ~5 consecutive infective bites (figure 1).

Protection-associated end points. The post–radical cure events and end points considered here are illustrated in figure 1. For the evaluation of each end point, subjects absent from the village for >7 consecutive days were considered to be lost to follow-up. Time to reappearance of parasitemia, determined as the time to the first positive blood smear during the period of weekly monitoring, was calculated by use of the day after the end of treatment as day 0. For this outcome, during the 11-week parasitological monitoring period, 8 of the 110 subjects enrolled on day 0 were lost to follow-up, 95 presented with parasitemia, and 7 presented with no blood parasites. The shortest time to the first positive blood smear was 7 days (1

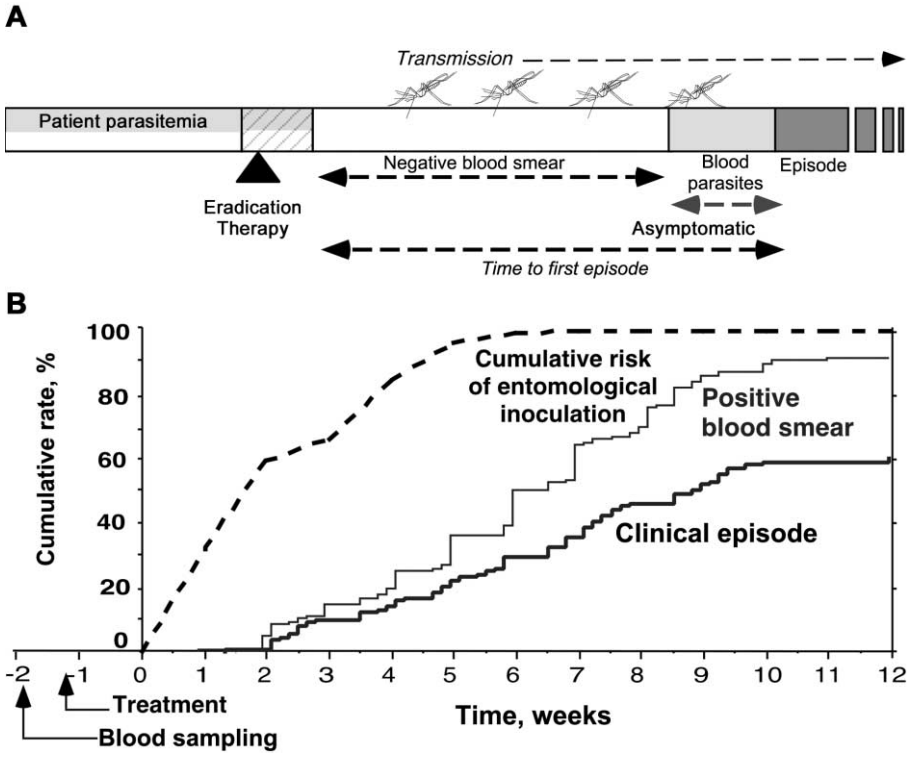

Figure 1. *A,* Schematic representation of the various end points investigated during longitudinal follow-up, after eradication therapy. Time of negative blood smear defines the time to reappearance of *Plasmodium falciparum* parasitemia, and this time added to the interval from the first positive blood smear to the first clinical access defines the time to the first malaria episode. Possible subsequent additional clinical episodes are not illustrated. *B,* Observed time-dependent ascending proportion, expressed as a percentage, of individuals with positive blood smears and with a confirmed clinical episode, after radical quinine cure administered during week 1 of the post–radical cure study. Time 0 was the last day of treatment. The cumulative risk of reinfection is calculated from the entomological inoculation rate monitored throughout the period.

individual, 20.6 years old, without occurrence of clinical access). Duration of asymptomatic carriage was calculated as the time from the detection of blood forms to the diagnosis of a clinical malaria episode, in days. For this end point, during the 21-week clinical monitoring period, 3 of the 95 subjects who tested positive by microscopy were lost to follow-up, 64 presented with at least 1 malaria episode, and 28 had no malaria episodes. Time to first malaria episode was the time from day 0 to the day on which a clinical malaria episode was diagnosed. For this outcome, during the 21-week clinical monitoring period, 11 of the 110 subjects included on day 0 were lost to follow-up, 65 presented with at least 1 malaria episode, and 34 had no malaria episodes (1 subject presented with a malaria episode during the last 10 weeks of clinical monitoring but did not present with parasitemia during the 11-week parasitological monitoring period). A total of 118 individual clinical malaria episodes were recorded during the 21-week follow-up. The incidence rate of clinical malaria episodes (per 1000 person-days) was 16.3 for the 0–14-year-old age group ($n = 53$ episodes for 53 children in this age group), 6.3 for the 15–29-year-old age group ($n = 29$ episodes for 29 subjects in this age group), and 2.2 for the ⩾30-year-old age group ($n = 5$ episodes for 28 subjects in this age group) ($P < .001$, Wald test).

Antigens and antigen preparation. The schizont, merozoite, and IRBC-membrane extracts were prepared from synchronous FCR3 parasites cultivated in candle jars in O^+ erythrocytes with 10% human serum, as described elsewhere [19, 38]. In brief, schizonts concentrated to ~95% were lysed in an equal volume of sterile, distilled water. Merozoites, collected after stepwise centrifugation at 2000 *g* and 4000 *g*, were washed 3 times in sterile PBS, were counted, and were frozen. Parasitized ghosts (IRBC membranes) and control ghosts (RBCs) were prepared as described by Fairbanks et al. [39]. The preparations were kept at −80°C in aliquots. Total protein concentration was estimated by use of the Coomassie blue Bio-Rad protein assay (Bio-Rad GmbH).

Immunograde synthetic $[NANP]_{10}$ was purchased from Neosystem. The glutathione-S-transferase (GST) fusion proteins R23 [31], PfEB200 [30], and PfEMP3-cl5 (previously called GST-5 [35]) and carrier GST, produced in *Escherichia coli,* were purified on glutathione agarose [28, 30, 31]. PfEB200, a GST fusion protein, and $MSP\text{-}1_{19}$, the C-terminal domain of protein encoded by the Palo Alto MSP-1 allele, produced in *Sodoptera frugiperda* cells infected by recombinant baculovirus and purified by metal agarose chromatography [40], were provided by D. Mattei and S. Longacre (Institut Pasteur, Paris), respectively. All proteins were found to be ⩾95% pure, as assessed by Coomassie blue-staining of SDS-polyacrylamide gels.

ELISA. Crude parasite extracts (10 μg/mL) were used to coat MaxiSorp plates (Nunc). PfEMP3-cl5 and PfEB200 R23 (1 μg/mL) were used to coat Immulon-4 plates (Dynatech). GST (0.7 μg/mL) was used to coat plates. $MSP\text{-}1_{19}$ (0.5 μg/mL) and $[NANP]_{10}$ (2 μg/mL) were used to coat Immulon-4 plates. Plasma samples were tested in duplicate, at a dilution of 1:200. Assays were performed as described elsewhere [19, 35, 41, 42].

A negative control (a pool of nonimmune serum samples from European subjects) and a positive control (a pool of serum samples from clinically immune adults living in Dielmo and Ndiop) were included in each assay. The background signal obtained by use of a control, uninfected red-ghost extract was deduced from the IRBC-membrane signal. The optical density (OD) for the GST carrier was subtracted from that for the GST-fusion protein. As described elsewhere [35], the IgG response to GST was weak. Results are expressed as OD ratios: $OD_{test\ sample}$:$OD_{control\ pool}$ [19, 41]. Antibody levels were considered to be statistically significant at $P < .05/n$, where n is the actual number of comparisons made.

Statistical analysis. The relationships between each end point and the immunological variables were tested by use of Kaplan-Meier estimated function (group differences evaluated by use of log-rank test) and Cox proportional hazards models. For the association of reappearance of blood forms with antibody levels, Akaike's information criterion was used to compare models, including immunological data analyzed dichotomously or continuously.

The possibly confounding effect of age was considered separately for each of the 4 end points. Optimal age groups were identified by use of Akaike's information criterion for each outcome, and, in the multivariate analysis, age stratification was made on this basis. The age of 15 years was used as the cut-off point for the significant decrease in the incidence of clinical episodes, and the age of 30 years was used as the cut-off point for low-density asymptomatic carriage. This stratification was consistent with the age-dependent distribution of the parasitological and clinical data that were available for this site, as a result of the long-term longitudinal follow-up [17] (A. Spiegel, L. Marrama, A. Tall, and J. F. Trape, unpublished data). For all models, first-level interactions between variables were checked (in particular, age and IgG responses) and were included in the model if significant.

A Poisson regression model was used to analyze the relationship between antibody responses and the incidence of malaria episodes during the 21 week follow-up. The optimal age group was identified by use of Akaike's information criterion. Three age groups (0–14, 15–29, and ⩾30 years) were used in the analysis. Malaria episodes were considered to be independent if separated by >15 days. The follow-up time was adjusted for individuals who received antimalarial treatment, by subtracting either a period of 15 days after diagnosis of the previous malaria episode or the first day of treatment from the days at

risk. Statistical analyses were performed with Egret (version 3.01; Cytel) and Statview (version 5.0; SAS Institute) software.

RESULTS

Prevalence and levels of antibody responses to parasite extracts and defined antigens. The seroprevalence of antibody and the levels of IgG directed against the various antigens are shown in table 1. The seroprevalence of antibody reactive with the 3 crude extracts was consistently high: the highest OD ratios were obtained with the schizont extract. The distribution of IgG within the population was similar for the 3 crude extracts, with coinciding mean and median OD ratios for each extract. The seroprevalence of antibody directed against PfEMP3-cl5 was 60%, and IgG levels were high in the responders. The seroprevalence and levels of antibody directed against R23 and PfEB200 were moderate, confirming previous observations made in this village [35]. The seroprevalence of antibody directed against MSP-1_{19} was high, and levels of these antibodies were particularly high (mean OD ratio, ~8). The CS-derived $[NANP]_{10}$ repeats were recognized by 50% of the individuals. Age was positively correlated with OD ratios for antibody against all antigens ($\rho = 0.37$–0.54; $P < .01$), with the exception of R23 (table 1). There was a positive association between some antibody levels and presence of parasites before eradication therapy. Subjects with positive thick blood smears during the 2 months preceding enrollment had significantly higher levels of IgG to schizont ($P = .015$), IRBC ($P = .04$), and MSP-1_{19} ($P = .03$).

Several antibody responses showed a significant degree of colinearity (table 2). A very strong correlation ($\rho \geq 0.8$; $P < .0001$) was observed between IgG against merozoite and schizont extracts and between IgG against schizont and IRBC-membrane extracts, probably reflecting the presence of common antigens. A similarly strong correlation was observed between IgG specific for merozoite and IRBC-membrane extracts, possibly reflecting cross-contamination and/or a colinearly acquired response. A substantial correlation ($\rho = 0.5$–0.8) was observed for IgG specific for MSP-1_{19}, PfEMP3, and PfEB200 and IgG reactive with the 3 parasite-derived extracts. The antibody responses to PfEMP3 and PfEB200 were correlated, and these responses were correlated to a lesser extent with MSP-1_{19}. Since PfEMP3, PfEB200, and MSP-1_{19} have no known structural similarity and do not elicit cross-reacting antibody, we interpret these findings as indicating that there is a tendency for antigen-specific responses to be acquired colinearly. The antibody response to R23 was not significantly related to any of the other antibody responses studied, including the response to parasite extracts. The antibody response to $[NANP]_{10}$ was significantly correlated with the antibody response to uninfected and infected erythrocyte membrane extracts, suggesting a parasite-independent association. On the whole, the noncolinear IgG responses that could be considered in a multivariate analysis were those to $[NANP]_{10}$, R23, and the IgG response to 1 of the other antigens, with the exception of PfEB200.

Relationship between antibody responses and reappearance of parasitemia (time to first positive blood smear). The cumulative reinfection rate for grouped individuals responding to a particular antigen was analyzed by use of Kaplan-Meier survival estimates. Various groups of responders were included in the model, defined according to the cut-off point for OD ratio selected on the basis of Akaike's information criterion for each antigen. A significant relationship between antibody response and reappearance of parasitemia was observed for all antigens investigated, with the exception of R23 and $[NANP]_{10}$

Table 1. Level and incidence of pre–radical cure IgG responses to parasite extracts and individual antigens, in 110 villagers from Ndiop, Senegal.

		OD ratio[a]			Correlation with age[c]	
Antigen	OD, mean ± SD	Mean ± SD	Median	Responders, %[b]	*P*	ρ
Merozoite extract	0.86 ± 0.49	3.1 ± 1.2	3.1	76	<.0001	0.52
Schizont extract	0.83 ± 0.43	5.5 ± 2.8	5.8	85	<.0001	0.54
IRBC membrane	1.01 ± 0.56	4.0 ± 2.0	4.0	79	<.0001	0.53
PfEB200	0.34 ± 0.67	2.8 ± 3.9	1.1	29	<.0001	0.4
PfEMP-3-cl5	0.79 ± 0.93	6.7 ± 7.1	2.9	60	<.0001	0.43
R23	0.12 ± 0.20	1.9 ± 1.5	1.6	27	NS	NA
MSP-1_{19}	0.72 ± 0.67	7.8 ± 6.6	4.7	77	.0001	0.37
$[NANP]_{10}$	0.27 ± 0.17	2.5 ± 1.3	2.0	50	.0003	0.35

NOTE. IRBC, infected red blood cell; MSP, merozoite surface protein; NA, not applicable; NS, not significant; OD, optical density; PfEMP, *Plasmodium falciparum* erythrocyte membrane protein.

[a] Ratio of $OD_{test\ sample}$:$OD_{control\ pool}$; background signals for IRBC and glutathione-S-transferase fusion proteins were subtracted from the antigen-specific individual signal.

[b] Subjects with an OD ratio >2.

[c] Association of IgG response with age of individuals, calculated by use of Spearman's rank correlation test.

Table 2. Analysis of colinearity of individual antibody responses to the various antigens studied.

	Relationship between antibody responses and antigens[a]						
Antigen	Merozoite	Schizont	IRBC	PfEB200	PfEMP3-cl5	R23	MSP-1_{19}
Schizont							
ρ	0.84						
P	<.0001						
IRBC membrane							
ρ	0.8	0.88					
P	<.0001	<.0001					
PfEB200							
ρ	0.47	0.56	0.59				
P	<.0001	<.0001	<.0001				
PfEMP3-cl5							
ρ	0.51	0.62	0.6	0.59			
P	<.0001	<.0001	<.0001	<.0001			
R23							
ρ	NA	0.22	NA	0.35	NA		
P	NS	.02[b]	NS	.0002	NS		
MSP-1_{19}							
ρ	0.67	0.72	0.67	0.44	0.4	NA	
P	<.0001	<.0001	<.0001	<.0001	<.0001	NS	
$[NANP]_{10}$							
ρ	0.27	0.27	0.47	0.27	NA	NA	NA
P	.005[b]	.006[b]	<.0001	.006[b]	NS	NS	NS

NOTE. IRBC, infected red blood cell; MSP, merozoite surface protein; NA, not applicable; NS, not significant ($P > .05$); PfEMP, *Plasmodium falciparum* erythrocyte membrane protein.

[a] Relationship between IgG response to the various antigens, calculated by use of Spearman's rank correlation test.

[b] Not significant after Bonferroni correction.

(figure 2). For IRBC membrane, PfEB200, and PfEMP3-cl5, this association was observed regardless of levels of IgG, with an OD ratio of $\geqslant 2$ ($P \leqslant .02$) (figure 2*C*– 2*E*, respectively). In contrast, only strong levels of IgG, namely OD ratios greater than the median value, were associated with the reappearance of parasitemia for the merozoite and schizont extracts and MSP-1_{19} ($P < .01$) (figure 2*A*, 2*B*, and 2*G*, respectively).

Since age was identified as a major confounder, we analyzed the relationship between antibody responses and the reappearance of parasitemia, using an age-stratified Cox proportional hazards model (table 3). The antibody responses to IRBC-membrane (hazard ratio [95% confidence interval /CI/], 0.889 [0.79–0.99], for 1 unit of OD ratio; $P = .049$) and to PfEMP3-cl5 (hazard ratio [95% CI], 0.956 [0.93–0.99], for 1 unit of OD ratio; $P = .008$) were significantly associated with the reappearance of parasitemia. Individuals without antibody responses to IRBC membrane or PfEMP3-cl5 had a 1.3- and 1.4-fold higher age-adjusted relative risk of reinfection, respectively, than did individuals with antibody responses at the median level.

Relationship between antibody responses and occurrence of the first clinical episode. Two independent analyses were conducted to assess the association between specific IgG responses and occurrence of the first clinical malaria episode. We first analyzed the duration of asymptomatic carriage, defined as time between the reappearance of parasitemia and the occurrence of the first clinical episode, in days. Using an age-stratified Cox regression model, we identified the antibody response to anti-$[NANP]_{10}$ as the sole factor significantly associated with the duration of asymptomatic carriage (hazard ratio [95% CI], 0.753 [0.59–0.96]; $P = .023$) (table 3). Individuals without anti-$[NANP]_{10}$ antibody had a 1.33-fold (95% CI, 1.04–1.70-fold) higher age-adjusted daily relative risk of clinical malaria than those with antibody to this antigen.

We analyzed time to first clinical episode, an end point used in some vaccine trials, separately [43]. This end point corresponds to the post–radical cure time for which the subject is free of symptomatic malaria, combining the time to reappearance of parasitemia and the duration of asymptomatic carriage.

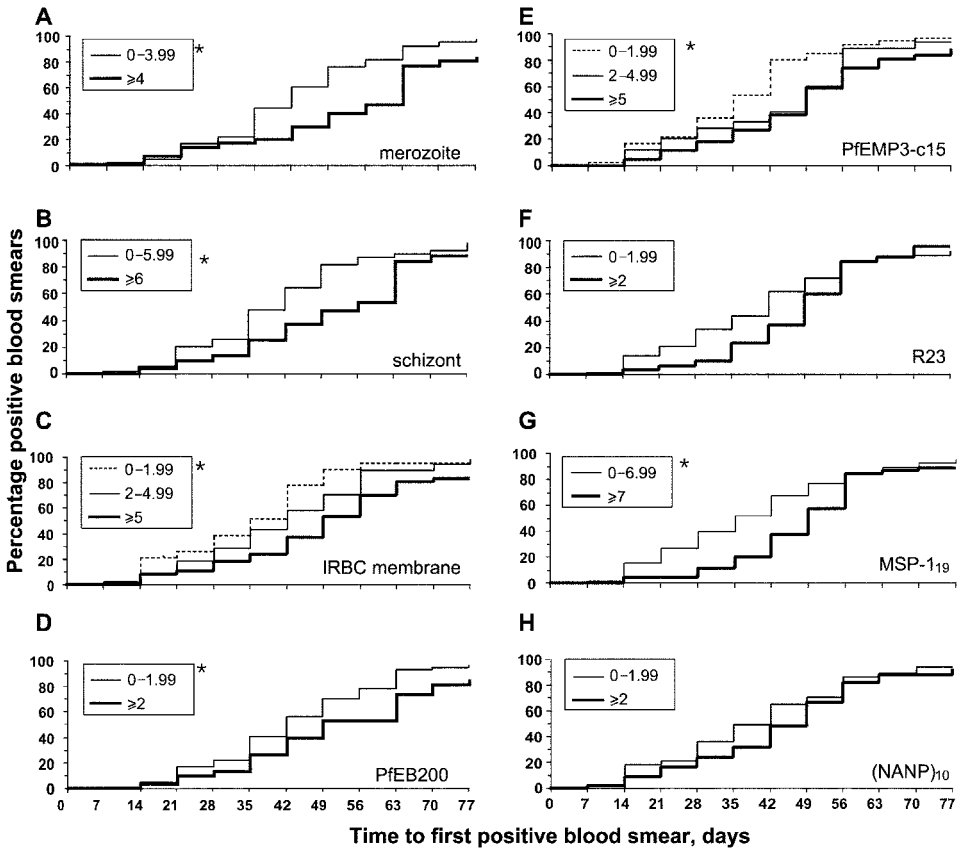

Figure 2. Kaplan-Meier ascending curves for the proportion of individuals with positive blood smears, established from weekly peripheral blood smears after eradication therapy, according to levels of antibody against merozoite extract *(A),* schizont extract *(B),* infected red blood cell (IRBC) membrane *(C),* PfEB200 *(D), Plasmodium falciparum* erythrocyte membrane protein 3-cl5 (PfEMP3-cl5) *(E),* R23 *(F),* merozoite surface protein–1_{19} (MSP-1_{19}) *(G),* and $[NANP]_{10}$ *(H).* The antibody levels in *A, B, D,* and *F–H* were dichotomized as negative *(thin line)* and positive *(thick line)* responders. In *C* and *E,* 3 antibody levels were considered: negative *(dashed line),* moderate *(thin line),* and high *(thick line).* $*P < .05$.

Time to first clinical episode was significantly associated with antibody response to parasite crude extracts. The lowest hazard ratio obtained was that for antibody directed against merozoites: 0.747 (95% CI, 0.59–0.95) (table 3).

Relationship between antibody responses and incidence of malaria episodes. We used a Poisson regression model to investigate the relationship between antibody responses and the incidence of clinical malaria episodes during follow-up. IgG responses to the merozoite extract (incidence rate ratio [95% CI], 0.823 [0.70–0.97]; $P = .019$), to the schizont extract (incidence rate ratio [95% CI] , 0.918 [0.85–0.99]; $P = .022$), and to R23 (incidence rate ratio [95% CI], 0.801 [0.65–0.98]; $P = .04$) were found to be significantly correlated with the incidence of clinical malaria episodes (table 3).

DISCUSSION

We have reported here the pattern of antibody responses to a panel of antigens and its relationship to resistance to reinfection and clinical protection, on the basis of precisely assessed longitudinal parasitological and clinical records for subjects from a rural Senegalese community. Distinct antibody responses were associated with each of the 4 protection-related end points considered. These responses could serve as markers of acquired protection, which impairs specific steps of progression from infection to disease.

Many studies have investigated the relationship between specific immune responses, whether humoral or cellular, and protection, and significant associations have been observed in prospective longitudinal studies in some settings [1–5, 14, 43]. The associations identified here are novel, partly because, of the antigens used here, MSP-1_{19}, $[NANP]_{10}$, and the schizont crude extract are the only antigens that have also been explored by other investigators. None of these antigens had previously been used to investigate an association with the 4 end points studied here. Furthermore, our work differs from the longitudinal immunoepidemiological studies described to date, in several respects. First, we conducted this study at the village level, in a well-documented setting in which the entire population had been involved in a demanding but highly informative longi-

Table 3. Age-adjusted association of IgG antibody responses to a panel of *Plasmodium falciparum*–derived antigens with specific infection end points.

	Relationship of specific antibody response with:							
	Reappearance of parasitemia[a]		Duration of asymptomatic carriage[b]		Time to first clinical malaria episode[c]		Incidence of clinical malaria[d]	
Antigen investigated	HR (95% CI)	P[e]	HR (95% CI)	P[e]	HR (95% CI)	P[e]	Incidence rate ratio (95% CI)	P[f]
Merozoite extract	NA	.075	NA	.118	0.747 (0.59–0.95)	**.016**	0.804 (0.68–0.95)	**.008**
Schizont extract	NA	.065	NA	.075	0.886 (0.80–0.98)	**.019**	0.967 (0.84–0.97)	**.008**
IRBC membrane	0.889 (0.79–0.99)	**.049**	NA	.052	0.822 (0.70–0.96)	**.016**	NA	.091
PfEB200	NA	.246	NA	.144	NA	.073	NA	.095
PfEMP3-cl5	0.956 (0.93–0.99)	**.008**	NA	.746	NA	.076	NA	.126
R23	NA	.057	NA	.419	NA	.097	0.791 (0.64–0.98)	**.029**
MSP-1_{19}	NA	.093	NA	.394	NA	.177	NA	.071
$[NANP]_{10}$	NA	.976	0.753 (0.59–0.96)	**.023**	NA	.186	NA	.526

NOTE. Significant ($P < .05$) correlates are indicated in bold type. CI, confidence interval; HR, hazard ratio; IRBC, infected red blood cell; MSP, merozoite surface protein; NA, not applicable; PfEMP, *P. falciparum* erythrocyte membrane protein.

[a] Time from day 0 after radical cure to first positive blood smear. Analysis was performed for 110 individuals; age was stratified as <15 and ⩾15 years old.

[b] Time from first positive blood smear to first clinical access. Analysis was performed for 95 individuals; age was stratified as <30 and ⩾30 years old.

[c] Time to first clinical episode (i.e., time from day 0 after radical cure to the day of the first clinical episode). Analysis was performed for 110 individuals; age was stratified as <15 and ⩾15 years old and as <30 and ⩾30 years old.

[d] No. of clinical malaria episodes experienced during the 21-week active case-detection study.

[e] Calculated using Cox proportional hazards models.

[f] Calculated using a Poisson regression model.

tudinal surveillance program during the preceding 4 years and have continued to actively participate. Second, we used end points of infection corresponding to successive steps in the progression from inoculation to disease. These end points are similar to those used for phase 3 trials of vaccine candidates targeting the erythrocytic phase, such as SPf66 [44, 45], but differ from those used in most seroepidemiological surveys investigating protection against morbidity [1–5]. Third, we conducted thorough follow-up, with daily clinical surveillance, as opposed to the weekly active surveillance used in most studies [2, 3, 5, 46, 47] or the passive case detection used elsewhere [9]. Parasitological surveillance was performed weekly, as in some other studies [6, 14], and was therefore more precise than the fortnightly [7] or monthly surveillance [3, 46, 47] performed in other studies. Finally, the use of antimalarial drugs is particularly well controlled in the population in Ndiop. This is a major confounding variable that is difficult to assess, because antimalarials are frequently used to treat febrile illness in rural Africa [18]. The Ndiop survey was designed such that access to antimalarial drugs was limited to the medical facility and was strictly controlled. Thus, our study design ensures precise and reliable measurement of the 4 malariometric indices considered.

Most immunoepidemiological studies conducted to date have sought to identify targets of acquired protective immunity. These studies have been based on the notion that the association between an immune response (cytokine secretion, presence of surface-reactive antibody or of antibody-promoting antibody-dependent culture inhibition, or parasite opsonization) and resistance to malaria identifies vaccine candidates. We had a different aim. We did not look for serological correlates of an effector mechanism. Instead, we looked for immune responses associated with acquired protection, regardless of the actual role of those responses in arresting parasite development, promoting parasite clearance, or preventing clinical manifestations. This is illustrated by the data on MSP-1_{19}. There was no evidence to suggest that levels of the anti–MSP-1_{19} antibody, as measured by ELISA, were protective. However, we cannot conclude from our results that the antibody response to MSP-1_{19} is unimportant, because the method used is not appropriate for investigating the role of the antibody response to MSP-1_{19} in protection. Indeed, our ELISA does not distinguish antibodies that inhibit merozoite invasion from antibodies without inhibitory activity [13, 48], the fine balance of which is critical in the control of parasite growth. Our data show that the antibody response to MSP-1_{19}, as measured by ELISA, is not associated with any of the end points studied here. The same holds true for the other negative results obtained. Similarly, we cannot conclude from our data that any of the IgG responses associated with a given end point has a direct role in protecting the individual. We simply tried to identify significant direct relationships of the antibody, as assayed here, with one or another protection end point.

The observed time to reappearance of blood forms departed substantially from the calculated cumulated risk of infection, reflecting the barriers encountered by the parasite to reach the

microscopic detection level. These barriers are a composite of physiologic obstacles and immune mechanisms arresting pre-erythrocytic development and/or restricting erythrocytic multiplication below the microscopic detection level. Kaplan-Meier analysis of immune correlates for the delayed reappearance of parasitemia showed 2 types of individual associations: (1) with presence of antibody to IRBC membrane, PfEMP3-cl5, and PfEB200, regardless of their level; and (2) with high levels of antibody to merozoite and schizont extracts and MSP-1_{19}. Whether this reflects a direct implication of these immune responses to the reinfection rate is uncertain. Two of the associations, namely antibody to IRBC membrane and antibody to PfEMP3-cl5, remained significant in the age-adjusted Cox models. It is tempting to speculate that, in addition to pre-erythrocytic immunity—which, in other studies, has been associated with delayed reinfection rates [6, 7]—immunity to parasite blood stages and/or to antigens expressed both at the pre-erythrocytic and the blood stages (such as PfEMP3) contributes to delaying the reappearance of blood forms and that, in this regard, the antibody level to some individual antigens is critical.

Protection against clinical episodes implies an ability to prevent parasites from reaching high blood-stage densities [18, 49]. Several recent studies have focused on the possibility that protection against clinical malaria involves the acquisition of a broad repertoire of antibodies directed against polymorphic or clonally variant surface antigens. Antibody responses to the variable domain of the merozoite surface antigen [4] (authors' unpublished data) and to the clonally variant neoantigens on the infected erythrocyte surface have been shown to be associated with protection against clinical malaria in African children [2, 3, 15] and against placental infection during pregnancy [50, 51]. However, since a large panel of strains/field isolates is required, it is difficult to use these responses in a convenient surrogate assay for naturally acquired protection.

We subdivided protection against clinical episodes into prolonged asymptomatic carriage and reduced incidence of morbid episodes. This delimited specific associations for each separate end point. It remains unclear whether the relationship between the IgG response to $[NANP]_{10}$ and the duration of asymptomatic carriage reflects a contribution of pre-erythrocytic immunity to delaying the occurrence of clinical malaria, by decreasing the probability of encountering (novel) strains successfully completing the hepatic phase, for example. It is also unknown whether the IgG response to $[NANP]_{10}$ detected in ELISA is directly involved in or merely reflects a colinearly acquired response. Overall, delay from inoculation to onset of malaria-attributable clinical symptoms was associated with IgG responses to crude extracts and was not simply the sum of the responses associated with time to reappearance of blood forms and duration of the asymptomatic phase. The number of malaria episodes experienced by an individual, which we intuitively assumed to be inversely related to his/her capacity to control a large number of strains, was associated with IgG reactive with the merozoite and schizont crude extracts and R23. This contrasts with the lack of association between IgG against schizont crude extracts and malaria morbidity seen in Gambian children [1]. This difference in results may be due to differences in study design, case-detection system, and definition of clinical episodes.

A major confounding factor in each of the 4 end points considered here was age. Most of the immunological responses measured were strongly age dependent. Several significant relationships disappeared after correcting for the effect of age. This was the case for the associations between IgG directed against MSP-1_{19} and delayed occurrence of first episode ($P = .02$) and with incidence of clinical episodes ($P < .001$), which were significant in univariate analysis and confirmed the results of other studies [7]. After adjusting for age, these associations were no longer significant. The only age-independent antibody response was that to R23, which was also unrelated to the other responses (table 2). Its association with reduced morbidity is particularly interesting in this context.

Methods based on the use of crude blood-stage antigens to measure the humoral antimalarial response are usually considered to reflect cumulative exposure to parasites rather than protection from malaria [1]. However, IgG directed against 1 of the crude extracts was associated, in an age-independent manner, with 3 of the 4 end points investigated. This finding is consistent with the observations made in Dielmo, where IgG, in particular IgG3, directed against schizont crude extract was found to be associated with protection against clinical malaria [42]. Further studies are required to determine whether the differences in results, particularly the lack of significant associations reported in other studies [1], reflect differences in case recruitment and/or definition. It should be noted that, in Marsh et al.'s pioneering study [1] of Gambian children in which protection against 1 or $\geqslant 2$ episodes was found to be associated with antibodies against IRBC neoantigens, the association between the anti-schizont ELISA response and protection against malaria morbidity was close to the limits of significance if a density cutoff point of 5000 parasites/μL was used to define a clinical episode ($P = .06$).

Overall, our results show that there are distinct and different immunological markers for different protection-related end points. It is tempting to conclude that impaired progression from inoculation to disease involves more than 1 immune response and multiple targets. Clearly, our results require confirmation in other settings in which malaria is endemic. The total panel of antigen that is exposed to the human immune system during parasite infection is very large. We have explored a very small fraction of the possible antigens, and no definitive answers can be provided at this stage. Nevertheless, we have shown that 2 end points critical for the assessment of inter-

vention methods—reinfection rate and morbidity—are associated with surrogate-specific immune responses that could easily be standardized, tested in other settings, and possibly used in large-scale epidemiological studies.

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