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Levels of Antibodies to *Plasmodium falciparum* Sporozoite Surface Antigens Reflect Malaria Transmission Rates and Are Persistent in the Absence of Reinfection

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Antibodies reacting with *Plasmodium falciparum* sporozoite surface antigens were measured by an immunofluorescence assay using wet preparations of sporozoites attached to poly-L-lysine-treated glass slides, a procedure which was found to be more specific than one using glutaraldehyde-treated and dried preparations. Subjects recovering from a first attack were found to be negative. In two African villages which differed in the level at which mosquitoes transmit the disease (1 and 100 infective bites per year and per individual), both the prevalence by age group and the levels of anti-sporozoite antibodies differed markedly, as follows. In the low-transmission area, these antibodies were not detected in subjects aged 2 to 10 years; thereafter, prevalence increased gradually with the age of the subject and reached 90% in subjects aged 50 to 80 years. In the high-transmission area, all of the subjects studied, including the younger ones, were positive. Anti-sporozoite antibody levels were independent of the levels of antibodies directed against blood stages. On average, the mean antibody titers were equal to 1/16 in the first village and 1/1,650 in the second one. These results suggest that stage-specific antibodies reflect the cumulative number of sporozoites inoculated in humans by mosquitoes and may therefore have useful epidemiological applications. In addition, the presence of stage-specific antibodies in the sera of African adults collected at different times after departure from the endemic area indicates that they may last for several years. During the course of this study, we observed a heterogeneity of immunofluorescence labeling in parasite populations prepared from mosquito salivary glands. This raises the question of possible qualitative or quantitative antigenic differences or both between one sporozoite and the other.

Experimental studies have demonstrated the high immunogenicity in humans and animals and the stage specificity of sporozoite surface antigens (3), but little data are available on the prevalence of naturally occurring anti-sporozoite antibodies. In individuals living in a hyperendemic area, it was observed that, despite the short life-span of this stage, specific antibodies could be detected and that their prevalence increased with the age of the subject (8). However, the significance of this finding could not be precisely determined and, surprisingly, no attempt was made since 1979 to correlate levels of stage-specific antibodies with other malariometric measurements.

Using a modified fluorescent antibody test performed on wet sporozoite preparations, we report here that antibody levels to stage-specific antigens correlate with the level of malaria transmission and reflect the cumulative sporozoite experience of exposed individuals. Furthermore, those antibodies can be long lasting, as they are still detected for several years in the absence of exposure to mosquito bite.

MATERIALS AND METHODS

Sera. To evaluate the specificity of the assay, we used 30 sera from healthy French blood donors, 12 sera from African adults living in a hyperendemic area, 4 sera from cases of accidental *Plasmodium falciparum* transfusion malaria, 2 sera from individuals infected by *P. vivax*, and sera from mice exposed several times to either *P. yoelii*- or *P. berghei*-infected mosquitoes. For titration of stage-specific antibodies, we studied, apart from the sera described above, 7 sera from *P. falciparum* primary attacks in European travellers, 8 sera from postimmune subjects (sera of African adults from hyperendemic areas collected at different times after departure from the endemic area), and two groups of 61 and 50 sera from individuals aged 2 to 80 years living in villages which were chosen because of their differences in levels of malaria transmission.

The group of 61 sera was collected in two villages located in a hypoendemic area 10 km from the Senegal River in the vicinity of Podor (northern Senegal). It is a very dry Sahel zone where rainfalls reached 225 mm during the year of the study. Captures of mosquitoes and dissection of salivary glands which were performed throughout the year showed that the transmission occurred from September to December and that, on average, each individual was bitten only once a year by mosquitoes harboring *P. falciparum* sporozoites (G. Parent, J. Vercruysse, N. Blanchot, R. Slavov, P. Gazin, P. Carnevale, J. C. Navdin, G. Delgado, and J. Roffi, Proc. 2nd Int. Congr. Malaria and Babesiosis, p. 192, Annecy, France, 1983).

The prevalence of *P. falciparum*-positive blood smears and -positive spleen rates varied from 12 to 17% during the year, and high parasitic loads were observed mainly in children less than 10 years old.

The group of 50 sera was collected in the village of Donsé, 50 km north of Ouagadougou, in Burkina-Faso. In this savanna area, rainfalls reach about 950 mm per year. Malaria transmission is seasonal. In the surrounding villages, an entomological study, performed by A. Hamon, showed that 0.9% of anopheles mosquitoes were infected; this fact, together with an average number of anopheline bites of 100

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per night and a mean 100 to 120 days of transmission, indicates that the number of infective bites per year and per individual is around 100. In both areas, sera studied were collected in the middle of the dry season (April and May).

Preparation of sporozoites. *P. falciparum* sporozoites were obtained by artificial feeding of *Anopheles stephensi* mosquitoes with blood from patients harboring gametocytes. Eight different batches, corresponding to eight different gametocyte donors, were used. *P. yoelii* sporozoites (17 × L strain) were prepared by feeding *A. stephensi* on infected mice.

To prepare antigen slides, several procedures were used. (i) Sporozoites from salivary glands were either untreated or fixed with 0.1% glutaraldehyde in cacodylate buffer as described by Nardin et al. (8), fixed with various concentrations of glutaraldehyde (1 to 0.001%) for 1 to 30 min, fixed in formaldehyde at 1%, or fixed in formaldehyde-glutaraldehyde mixtures in various proportions. Thereafter, sporozoites were deposited on glass slides and air dried, and slides were kept at −70°C with silica gel until use. (ii) In further experiments and for all immunofluorescence assay (IFA) titrations, sporozoites were used either fresh or glutaraldehyde fixed (0.05%) as wet preparations as follows. Slide wells were coated with 20 μl of a 1% solution of poly-L-lysine (molecular weight, 800,000; Sigma Chemical Co.) and dried at 50°C for 24 h. Thereafter, drops (1 μl each) of sporozoite suspensions (adjusted to about 50 sporozoites per μl) were deposited, and slides were kept at 4°C for 24 h in a moist chamber to allow attachment of sporozoites to the poly-L-lysine film. Slides were used for IFA the next day. Titrations of all sera from the African villages were made with a single sporozoite suspension from a single strain. Fixed sporozoites were kept at 4°C, and all titrations were performed within 3 weeks.

IFA. With all types of preparations, IFA was carried out with the first serum at various dilutions in phosphate-bufferedsaline (1/10 to 1/10,000) and fluorescein-conjugated anti-human immunoglobulin G (IgG), IgA, and IgM (Nordic) as the second serum diluted 1/100 in a 0.02% Evans blue solution (or anti-mouse immunoglobulin when pertinent). Each experiment included, apart from known positive and negative controls, a transfusion malaria serum (diluted 1/100) to check the specificity of the sporozoite preparation used.

When wet sporozoite preparations were used, serum dilutions (50 μl) were added slide by slide; care was taken to ensure that no well of the slide would dry during the procedure. Washings (10 min each, repeated three times) were performed by slow immersion of the slide in phosphate-buffered saline. At the end of the procedure, slides were mounted with cover slips in a 70% phosphate-buffered saline–30% glycerol mixture and examined under ×500 and ×1,000 magnifications by epifluorescence with a Leitz microscope. Nonfluorescent sporozoites were searched for under white light. Antibodies to blood stages were measured by an IFA on acetone-fixed thin smears of *P. falciparum* in vitro cultures.

RESULTS

Specificity of the IFA. With all fixation procedures tested which included a final step of air drying of the sporozoites, interstage and interspecies cross-reactions were observed. When 0.1% glutaraldehyde-fixed sporozoites were used, sera from four transfusion malaria cases reacted at high titers (i.e., titers of 1/600, whereas IFA titers using blood stage antigens were 1/1,800). Absorption of these sera on *P. falciparum* blood schizonts decreased to a similar extent for titers of both stages. Sera from individuals infected by *P. vivax* and from mice infected by either *P. yoelii* or *P. berghei* were also found to be positive. Conversely, sera from *P. falciparum*-sensitized subjects reacted with *P. yoelii*-fixed and dried sporozoites, although at lower titers than those of the homologous antigen. Similar results were recorded with eight different sporozoite preparations, whatever the fixation procedure used. In each instance, surface fluorescence was more intense than that of the internal part of the parasite.

In contrast, when either freshly isolated or glutaraldehyde-fixed sporozoites were used in wet preparations, either in tubes or on poly-L-lysine-coated slides, only sera from African adults were positive (endpoint titer, 1/400 to 1/6,400), whereas those from subjects with blood-induced infection or heterologous infections were consistently negative (at dilutions of 1/50 and above).

In further experiments, titrations were performed with wet preparations of 0.05% glutaraldehyde-fixed sporozoites on poly-L-lysine slides. Fixation allowed the antigen to be kept for at least 6 weeks at 4°C, and results were similar to those of titrations performed on freshly isolated sporozoites.

Because weak positive reactions could be obtained with sera from healthy controls at a 1/10 dilution, or more rarely at 1/20 and 1/50 dilutions, the assay was considered to be specific at 1/100 and higher dilutions.

Levels of anti-sporozoite antibodies. The prevalence of antibodies to *P. falciparum* sporozoite surface antigens differed markedly in the two villages studied (Fig. 1).

In the village of Podor in northern Senegal, where malaria transmission is very low, sporozoite-specific antibody could not be detected in subjects aged 2 to 10 years. However, most of these subjects had been infected, as shown by a high prevalence (78%) of antibodies to blood stages (at titers up to 1/3,200) and by the presence of parasites in the blood of some of them. In the 10- to 15-years-old age group, antibodies to sporozoites were detected in 36% of the patients studied; with increasingly older age groups, prevalence increased but never reached 100%, even in subjects exposed for 50 to 80 years to infective bites.

In contrast, in Donsé, a village of the Burkina Savana where malaria transmission was estimated to be about 100 times higher, the prevalence of stage-specific antibodies was high, each of the 50 individuals studied (including the younger ones, 2 to 5 years old) having significant levels of antibodies.

The amount of stage-specific antibody developed by exposed individuals also differed, depending on the sporozoite load, as shown by the difference in average titers obtained in the two areas studied and by an age-dependent increase of titers. In the village with low endemicity, titers are low, most of the positive ones being in the range of 100 to 400, whereas in the zone of higher endemicity, more than half of the subjects more than 50 years old have titers in the range of 3,200 to 6,400, and more than 80% of the younger subjects have antibody titers of >800.

Table 1 shows the geometric mean of anti-sporozoite antibody titers by age group in each village. In the dry area, there is a progressive and regular age-related increase of antibody levels. In the savanna area, high antibody levels are present without major variation in children, and an increase occurs in the two groups of older subjects. A slight decrease is even observed in young adults (15 to 25 years old); this may correspond to the fact that this part of the population migrates to find work in large cities of Africa (or of Europe) where they are generally less exposed to the disease. On

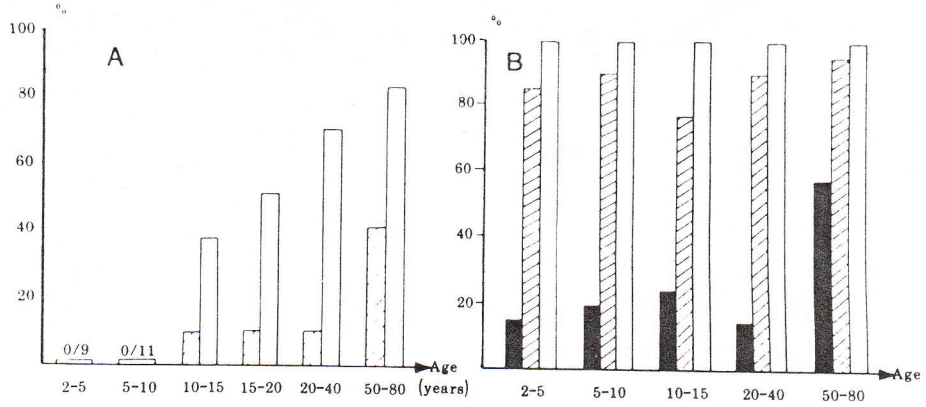

FIG. 1. Prevalence of antibodies to sporozoite surface antigens in the low-transmission area of northern Senegal (1 infective bite per year) (A) and in the high-transmission area of Burkina-Faso (100 infective bites per year) (B). ▭, Total prevalence (titers, ≥1/100); ▨, prevalence of titers of ≥1/800 to 1/1,600; ■, prevalence of titers = 1/3,200 to 1/6,400.

average, the geometric mean of titers of all subjects studied in each area differs by a factor of 100.

Seasonal variations of anti-sporozoite antibodies were estimated in Podor by titrating serum samples collected in May and in December from the same individuals. No seasonal difference in either prevalence or titer was noted among the 16 patients followed up (data not shown). This result is in agreement with the long remanence of those antibodies (see below).

The comparison of levels of antibody reacting with the sporozoite surface antigens and those reacting with asexual blood stages showed that they behave independently (r = 0.29 in Podor and 0.07 in Donsé); this indirectly confirms the fact that anti-sporozoite surface antibodies are highly stage specific. In Podor, 80% (26 out of 32) of individuals negative for sporozoite antibodies had detectable antibodies to blood stages. Interestingly, in 8 of the 50 cases studied in Donsé, a low to negative titer of antibodies to blood stages (geometric mean, 1/125) coexisted with much higher titers of antibodies to sporozoite surface antigens (range, 1/400 to 1/6,400; geometric mean, 1/1,600).

Antibody levels in primary attack cases and postimmune individuals. Of 21 sera from seven European subjects who experienced a *P. falciparum* primary attack after a short visit to an endemic area, all were negative with wet sporozoite preparations (at 1/100 and even 1/50 dilutions). Each of these individuals developed high titers of antibodies to blood stages (four cases at 1/1,800, two cases at 1/5,400, and one case at 1/16,200). Because these subjects were presumably exposed to very limited numbers of infective bites, possibly only one, the results suggest that a single infection, regardless of how many sporozoites are injected, does not induce detectable levels of antibodies. These results are in agreement with those obtained with sera from the children of Podor.

Titration of sera from African adults whose last exposure to bites of infected mosquitoes varied from a few months to 11 years suggests that, even in the absence of antigenic restimulation, antibodies specific for sporozoite surface antigens are produced at detectable levels for many years (Table 2). All subjects except two were found to be positive for antibodies to both sporozoites and asexual blood stages. Moderate or high levels of antibodies to blood stage antigens are found for many years, as observed previously in many of the African subjects residing in Paris (unpublished observations).

No clear correlation (i.e., decrease) between the levels of anti-sporozoite antibodies and the length of stay away from an endemic area was observed. However, those individuals came from areas with presumably different transmission levels. The initial amount of antibody before arrival in the nonendemic area is not known, precluding any appreciation

TABLE 1. Geometric mean of IFA titers by age group in the low (Podor)- and high (Donsé)-transmission areas

Area	Geometric mean of IFA titer of the following age group (in yrs):						Mean antibody titer of the area
	2–5	5–10	10–15	15–25	25–50	>50	
Podor	0	0	7	28	98	288	16
Donsé	1,572	1,481	1,312	1,055	1,600	2,377	1,650

TABLE 2. Levels of antibody to blood stage and sporozoite surface antigens in two groups of patients

Patient group (patient no. or length of stay)	Level of antibody to:	
	Asexual blood stages	Sporozoite surface antigens
Primary attack[a]		
1	5,400	0
2	5,400	0
3	1,800	0
4	1,800	0
5	1,800	0
6	16,200	0
7	1,800	0
Postimmune[b]		
1 mo	1,800	800
2 mo	1,800	800
6 mo	1,800	100
3 yr	1,200	400
3 yr	600	100
3 yr	600	0
6 yr	400	800
6 yr	1,800	200
8 yr	1,800	400
10 yr	1,200	800
11 yr	600	0

[a] Primary attack, first *P. falciparum* attack in European travellers (21 sera studied at different times after treatment of seven patients; only the maximum titer recorded is shown).

[b] Postimmune, length of time in France without known return to a malaria-endemic area.

of a possible decrease. Therefore, no conclusion can be drawn from low or negative titers observed 6 months to 3 years after arrival.

The data suggest mainly that after several years of antigenic stimulation, when natural boosting is interrupted, anti-sporozoite antibodies may last for up to 6 to 10 years.

Heterogeneity of antibody binding to sporozoites. During the course of this study, differences in fluorescence labeling were observed from one sporozoite to the other. At high antibody concentrations, all sporozoites had a homogeneous bright surface fluorescence. However, when higher dilutions were screened, some sporozoites were weakly labeled, whereas others remained bright. At the last two to four dilutions before the endpoint, about 10 to 35% of the sporozoites attached to the poly-L-lysine film were totally negative. Similar observations were made among several sporozoite preparations. The percentages of negative or minimally reactive sporozoites varied from one serum to the other, but negative ones were scored with nearly all of the sera tested.

DISCUSSION

Sporozoites are injected in unknown but probably small numbers by infected mosquitoes and have a very short life-span in the human body. Some rapidly develop into liver stages with different antigenic features (5), whereas the remaining ones are probably taken up by macrophages, processed, and presented to the immune system.

The amount of antigenic material and the time of contact with immunocompetent cells are quite different for the blood stages which circulate in much larger numbers for weeks or months in individuals exposed in endemic areas. From this point of view, it is not surprising that all primary infections and all subjects who received a total of less than 10 infective bites were serologically negative for anti-sporozoite antibodies. In contrast, the high levels of antibodies reached in other subjects confirm results obtained with animal models, which demonstrated the high immunogenicity of sporozoite surface antigens (3). Results show that a single sporozoite inoculation does not induce antibodies detectable by the method used but that a single inoculation repeated every year would after 10 to 15 years at least induce detectable but low levels of stage-specific antibodies. Conversely, multiple inoculations per year induced a strong humoral immune response within 2 years in each of the individuals studied.

Thus, the amount of stage-specific antibodies developed seems to depend on the cumulative number of sporozoites inoculated. Similar titers could be reached either after a short time of exposure to high numbers of sporozoites or after many years of exposure to low numbers. As a matter of fact, the average titer of Podor adults exposed to an estimated 50 to 80 sporozoite inoculations over 50 years is six times lower than that of Donsé children exposed to a total of 200 to 500 sporozoite inoculations over 2 to 5 years.

The relationship between antibody levels and malaria transmission rate is supported by the following findings: a clear difference in prevalence between the two areas studied, which contrasts with only minor differences of prevalence of antibodies to blood stages; in the dry area, an increase in prevalence with age, that is, with the duration of exposure; an increase in prevalence of high antibody titers with age, in both areas; and a striking relationship between the inoculation rate ratio (1/100) and the ratio of geometric mean IFA titers (16/1,650). It remains to be determined whether an even greater rate of transmission, such as that found in a holoendemic area, would result in increased antibody production.

The data demonstrate the superiority in epidemiology of immunoassays exploring one or a limited number of highly specific antigens. The measurement of sporozoite-specific antibodies is clearly of no help in the diagnosis of individual malaria cases but is obviously very informative in the field of malaria epidemiology. Sporozoite indexes are seldom measured because of the time required for the capture of mosquitoes and for the dissection and examination by microscopy of salivary glands. The use of immunoassays recently purported (2, 9) to detect the presence of parasites in mosquitoes does not preclude the need to study the number of anopheline bites per day at different periods of the year and still requires manpower to separate anopheles from other mosquitoes and anthropophilic ones from others. Because of its cumulative aspect, the antibody response to sporozoite surface antigens appears to be a complementary, or possibly alternative, means to estimate malaria transmission in a given area. This parameter is an important component of the epidemiological picture of the disease. It may appear essential, when synthetic and recombinant vaccines will be tested, to evaluate the rate of natural challenge and the level of protection achieved by artificial or natural immunization or both.

The inverse relationship, found in some subjects from Donsé, between sporozoite antibody titers which were high and blood stage antibody titers which were low or undetectable suggests the presence of sporozoite-blocking antibodies in the latter subjects. We observed recently (7) that the degree of inhibition of sporozoite invasion in cultured hepatocytes was a function of the antibody concentration from mice immunized with recombinant circumsporozoite peptides. Of the sera from Donsé, 30% reacted as strongly with the sporozoite surface as those from the immunized mice tested.

In view of vaccination with circumsporozoite antigens, an interesting and unexpected finding is the long life-span of antibodies in postimmune subjects. If protection against sporozoite challenge can be obtained by immunization with sporozoite surface antigens, our results suggest that this protection could last for a long time without restimulation.

For future epidemiological applications, the availability of pure synthetic or recombinant peptides (1, 4, 6) should allow the operation of more simple quantitative assays such as the enzyme-linked immunosorbent assay or the radioimmunoassay. However, artificial peptides may not prove to be as appropriate as they seem at first. Using sera from immunized mice, we observed no correlation between enzyme-linked immunosorbent assay performed with the immunizing peptide and IFA performed with the authentic sporozoite protein (7). Until this question is resolved, the IFA remains an operational assay for epidemiological purposes. Our results confirm that internal sporozoite antigens are not stage specific. They indicate that, in contrast to previous reports (8), specific measurements are difficult to achieve with fixed and air-dried preparations, whereas wet preparations were reliable.

The heterogeneity of the fluorescence labeling from one sporozoite to the other that we observed with fixed, as well as unfixed, salivary gland sporozoites has not been reported in other IFA studies (8). However, it is consistent with several observations made with the circumsporozoite precipitation assay (3). The variable, and sometimes low, percentage of circumsporozoite precipitation assay-positive sporozoites suggests that, within a single preparation, some

parasites are less susceptible to the effect of antibodies than others. More recently, we observed variations from one sporozoite to the other of both circumsporozoite precipitation assay reaction and surface fluorescence in sporozoites incubated with mice antibodies (7). This may reflect either quantitative or qualitative differences in the surface antigen(s) but in any case has important consequences if the corresponding antibody is supposed to block the infection. Clearly, one major issue is whether minimally reactive sporozoites are truly infectious.

On the basis of results from competitive radioimmunoassays, it has been proposed that all strains of *P. falciparum* share the same circumsporozoite antigen (10). However, the possibility that some sporozoites from each isolate may have variant circumsporozoite antigens does not seem to have been investigated. The differences in the amino acid sequences of the circumsporozoite genes cloned by two different research groups (4, 6) may correspond to differences in the antigenicity of the sporozoite surface proteins.

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