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Evidence for Diversity of *Plasmodium falciparum* Sporozoite Surface Antigens Derived from Analysis of Antibodies Elicited in Humans†

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We have compared the reactivities of antibodies developed by individuals frequently exposed to *Plasmodium falciparum* infections with the epitopes contained within the repeats of the circumsporozoite (CS) protein and their reactivities with the epitopes of a native molecule(s) accessible on the sporozoite surface. Results of direct-binding assays and competition assays between artificial and native molecules or between human antibodies and anti-CS monoclonal antibodies suggest that humans respond preferentially to epitopes not contained within the repeats of the CS protein and probably not contained in the whole CS protein. Human monoclonal antibodies reactive with *P. falciparum* sporozoite surface antigens were produced by Epstein-Barr virus transformation of human lymphocytes. Their pattern of reactivity with sporozoites from a number of different isolates indicates the existence of several distinct epitopes on the parasite surface. Differences between isolates and between sporozoites within a given sample were observed. No single human monoclonal antibody capable of detecting an epitope expressed in all the parasites studied was found.

The circumsporozoite (CS) protein is generally considered the only antigen on the surface of malaria parasites in the sporozoite stage (11). However, it should be remembered that the identification and subsequent characterization of this antigen in several different malaria parasite species were direct consequences of the initial production of monoclonal antibodies (MAbs), and as such CS antigens were selected on the basis of their immunogenicity in mice. The main epitopes of the CS antigen of *Plasmodium falciparum*, as defined by these means, were found in a series of tandemly repeated tetrapeptides, $(NANP)_x$, and have been found in all isolates studied to date (13, 17). No detailed analysis of sporozoite antigenic content and immunogenicity in humans has yet been attempted; however, antibodies directed against NANP repeats have been found to be prevalent in human sera. Results from our previous studies (6, 10) have led us to suspect that quantitative or qualitative differences in the antigens may occur from one sporozoite to another. The patterns of reactivity observed between sporozoites and human sera suggested that naturally infected individuals had produced antibodies directed to a variety of epitopes on the sporozoite surface.

In the present study, we evaluated the correspondence between the specificities of antibodies occurring in humans exposed to frequent antigenic stimulation and the epitopes contained within the NANP repeat peptide, as well as another native molecule(s) accessible on the sporozoite surface.

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† This paper is dedicated to Richard L. Beaudoin, who died recently. For the past 20 years, occasionally running against the tide of popular opinion, Dick made essential contributions to the study of malaria, and his personal commitment was highly encouraging and rewarding to us all.

MATERIALS AND METHODS

Antigens. (i) Sporozoites. Sporozoites were obtained either from *Anopheles stephensi* fed on cultured gametocytes or from *Anopheles dirus* infected by membrane feeding on patient gametocytes. These *P. falciparum* strains included the NF54 strain, presumed to be from Africa; 3D7, an NF54-derived clone; 7G8, a clone from a Brazilian isolate; and 10 isolates from Thailand (1, 2, 5, and 6 from Kanchanaburi; 3 from Ranong; 4 from Rayong; 7 from Chantaburi; 8 and 9 from Trad; and 10 from Prachimburi).

(ii) CS peptides. To perform the immunoassays, we used the R32tet32 recombinant peptide (R32) composed of 30 NANP and 2 NVDP repeats (16); synthetic peptides consisting of either three or four NANP repeats (2) conjugated to bovine serum albumin, with the concentration expressed as the weight of the peptide alone; and a recombinant complete CS protein which was devoid of the repeat region and fused to 81 amino acids of the influenza virus nonstructural protein (D. M. Gordon, M. Hollingdale, M. Seguin, I. P. Schneider, C. Silverman, G. F. Wasserman, J. P. Verhave, D. Sylvester, T. Porter, and M. Gross, submitted for publication), hereafter referred to as repeatless CS peptide.

Antibodies. (i) Human sera. In order to study the specificities of antibodies occurring in exposed individuals, we used serum samples from various geographic areas with a wide range of reactivity to sporozoite surfaces, as measured in an indirect fluorescent-antibody test (IFAT): 18 serum samples were from high-transmission areas (Gabon, 2; Upper Volta, 6; Congo, 10; their titers ranged from 1,600 to 51,200) and 9 serum samples were from areas with lower transmission (northern Senegal, 3; Thailand, 2; French Guyana, 4; their titers ranged from 200 to 1,600).

(ii) Mouse sera. Sera directed against the R32 recombinant protein were raised in mice by administrating 50 μg of

protein, with Freund complete adjuvant, alum, or murabutide as an adjuvant (16).

(iii) MoMAbs. We used purified immunoglobulin G (IgG) from two mouse MAbs (MoMAbs), NFS1 (IgG1) and NFS2 (IgG2a), which recognize the *P. falciparum* CS protein (14) with IFA titers of 8×10^6 and 1×10^6, respectively.

(iv) Human MAbs (HuMAbs). Epstein-Barr virus transformation of peripheral lymphocytes from 18 Thai donors and 18 Gabonese donors was performed as previously described (5).

From 26 transformed lymphoblastoid lines that were IFA positive, 366 IFA-positive clones were derived, of which 29 were found to be both sufficiently positive and stable enough to be used. All of these antibodies belonged to the IgM class, and their fragility made it difficult to concentrate them, thereby limiting the scope and type of experiments that could be performed with them.

Immunoassays. (i) IFAT. The IFAT was performed as previously described by using wet preparations of parasites attached to poly-L-lysine (Sigma Chemical Co.; molecular weight, >500,000) films, to measure surface reactivity (6). Depending on the antibodies being studied, the revealing serum was either fluorescein isothiocyanate-labeled anti-human IgG, IgA, and IgM (Pasteur Production) or anti-mouse IgG, IgA, and IgM (Organon Teknika), both at a 1/200 dilution in 0.05% Evans blue solution in phosphate-buffered saline. The percentages of staining and nonstaining sporozoites were assessed by UV and phase-contrast microscopy. Preliminary studies had shown that the sensitivity of this wet sporozoite IFAT for surface antigens was comparable to that of the more classical assay using air-dried and acetone-fixed sporozoites. The titers of the two MoMAbs directed to CS protein repeats were found to be identical in the two assays.

Each experiment included the following controls: no serum, normal human serum (1/100), positive hyperimmune serum (1/200), MoMAb NFS1 (1/10,000), and a transfusion malaria serum (1/200); the latter two controls enabled us to ascertain the integrity of individual sporozoites and the surface specificity (transfusion malaria sera are negative in the wet IFAT and positive with internal antigens of sporozoites in case parasites were fixed or had dried during the assay).

(ii) Antigen competition assays. To a given dilution of each MAb or serum tested, synthetic or recombinant peptides were added at final concentrations ranging from 1 to 10^{-16} g/liter. Following overnight incubation at 4°C, the mixture was used in an IFAT and compared with the control antibody dilution without peptide. Since the individual MAbs and sera reacted over a wide range of concentrations (10^{-2} to 10^{-7}), all dilutions were adjusted to a fixed ratio of the endpoint titer (e.g., a 10× or 100× concentration would correspond to a 10^{-5} or 10^{-4} dilution, respectively, of an antibody with a titer of 10^{-6}).

(iii) Antibody competition assays. Competition between MoMAbs and human sera was assessed by incubating sporozoites with the MoMAbs, washing them three times, incubating them with a human serum, washing them three times, and revealing them with the anti-human conjugate. Controls were handled in the same way, except that the conjugate used was anti-mouse immunoglobulin. In complementary experiments, the order was reversed, with the first reagent being the human serum and the second being the MoMAb.

(iv) ELISAs. For solid-phase enzyme-linked immunosorbent assays (ELISAs), 96-well plates (Nunc Immunoplate II) were incubated with either R32tet32 or the repeatless recombinant CS peptide as a 10-μg/ml solution in carbonate buffer (pH 9.6). They were blocked with casein (Regilait; 2.5%)-phosphate-buffered saline (0.1 M; pH 7.2) buffer for 2 h. After being washed, sera were tested at a 1/100 dilution in blocking buffer supplemented with 0.05% Tween 20. After three washings, the reaction was revealed by use of peroxidase-labeled anti-human or anti-mouse immunoglobulin (Biosys; diluted 1/2,000) and *ortho*-phenylenediamine substrate. Readings were made at 492 nm with a Titertek Multiscan photometer. Each manipulation included as negative controls sera from 14 healthy French donors with no history of travel in malaria-endemic countries. Results are expressed as the ratio of the mean absorbance of duplicate wells from each tested serum to the mean plus 3 standard deviations of the 14 negative controls (or the mean from 5 normal mice for mouse sera).

Dot ELISAs were performed on nitrocellulose membranes (Schleicher & Schuell, Inc.; BA 85 401144), on which dots of 5 μl of a 0.1-g/liter dilution of the recombinant peptide were deposited. The membranes were then saturated with Tris hydrochloride–5% milk buffer (pH 8) for 1 h, incubated with a MAb or serum which had been diluted in blocking buffer at 100× or 10× its IFAT endpoint titer, washed, and incubated with either anti-human or anti-mouse antibodies labeled with peroxidase (Biosys; diluted 1/400), and the reaction was revealed with diaminobenzidine substrate. Competition dot ELISAs between MoMAbs and sera were also performed similarly to the IFAT.

(v) Western blots (immunoblots). One million *P. falciparum* NF54 sporozoites were obtained by dissection and grinding of salivary glands from infected *A. stephensi* mosquitoes. To the sporozoite pellet was added 4 volumes of sample buffer, and the mixture was boiled for 5 min. The sporozoite extract was electrophoresed in a 7.5% polyacrylamide gel containing sodium dodecyl sulfate, as described by Laemmli, and the gel was transferred to nitrocellulose (Schleicher & Schuell; BA85). Strips from the nitrocellulose filter were incubated in Tris hydrochloride (pH 8)–5% milk buffer, soaked, incubated with a 1/100 dilution of human sera in the same buffer, washed, and incubated with anti-human goat antibody labeled with ^{125}I. Autoradiographs were performed with Kodak X-Omat film with amplifying screens.

RESULTS

Comparison of IFA titers to sporozoite surfaces in the wet IFAT with results from Western blots performed with sporozoite extracts led us to suspect that additional sporozoite surface antigens may exist. Four of eight serum samples had both high IFA titers and detectable antibody to the CS protein and its precursor in Western blots. In contrast, the remaining four serum samples, which had similar IFA titers, either did not recognize the CS protein polypeptides (two cases) or reacted very weakly (two cases). Therefore, in four of eight cases, there was a striking discrepancy between recognition of the CS protein and levels of antibody reactivity with sporozoite surface molecules as determined by IFAT. It is worth noting that the latter sera potentially recognized other, non-CS proteins in sporozoite extracts (Fig. 1), whose surface location was not known.

To further investigate the correspondence between anti-CS antibodies and antigens detected on the surface of the parasite, several competition assays were carried out.

In antigen competition assays, MoMAb NFS-1 reactivity could be fully inhibited by very low concentrations of CS repeat peptides. At a 10× concentration of its endpoint titer

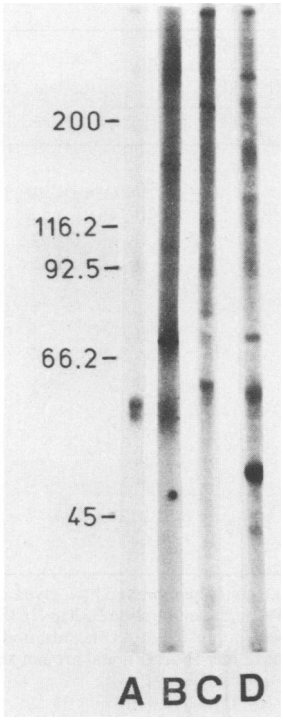

FIG. 1. Autoradiograph from Western blots of extracts from *P. falciparum* NF54 sporozoites. Sporozoites were probed by MoMAb NFS1 directed to CS protein repeats (A), human serum (IFA titer, 1/3,200) containing antibodies directed to the CS protein doublet (B), and two human serum samples (IFA titers, 1/6,400 [C] and 1/12,800 [D]) negative with the CS protein and its precursor. The serum samples were diluted 1/100, and the MoMAbs were diluted 1/100,000 and were revealed by ^{125}I-labeled second antibodies. Molecular sizes (in kilodaltons) are shown on the left.

of 8×10^{-6}, MoMAb NFS1 was no longer reactive with the sporozoite surface following preincubation with 10^{-14} g of the R32 recombinant peptide per liter (Fig. 2). The inhibition was concentration dependent, since at a 100× concentration, 10^{-9} g of the peptide per liter was necessary to reach complete inhibition (data not shown). MoMAb NFS2, which also recognizes the CS protein, showed a lower reactivity to R32, being markedly inhibited by 10^{-7} g of the peptide per liter and fully inhibited by a concentration of 10^{-4} g/liter (data not shown).

In contrast, competition assays performed with human sera instead of MAbs showed that the R32 antigen rarely competed efficiently with native sporozoite antigens for human antibodies (Table 1). The results obtained with 27 serum samples fall into three general categories: (i) serum samples whose sporozoite reactivity was blocked by rather small amounts of R32 (10^{-6} g/liter) (8% of the serum samples); (ii) samples whose reactivity was inhibited only by very high concentrations of the pure molecule, i.e., 10^{-3} to 10^{-1} g/liter was necessary to reach inhibition (52% of the serum samples); and (iii) samples with unaltered IFA reactivity following incubation with 10^{-1} g of the peptide per liter, the highest concentration tested in this experiment (40% of the serum samples). Complementary experiments were performed with seven serum samples in the latter category. The sporozoite reactivity could not be blocked by 1.0 g of the R32 peptide per liter added to a 10× concentration of the sera or by 0.5 g of peptide per liter added to the endpoint dilution (data not shown).

With the 12-mer $(NANP)_3$ and 16-mer $(NANP)_4$ synthetic peptides, similar results were obtained, except that higher concentrations of these smaller peptides were required to reach the same degree of inhibition. In 75% of the cases, no inhibition was observed at the highest concentration tested [10^{-1} g of $(NANP)_3$ or $(NANP)_4$ per liter] (Table 1).

The indication given by the IFA competition assays that antibody to the repeat peptide was not the major antibody specificity in most of the serum samples tested was confirmed by results of solid-phase ELISAs (Table 1), as well as

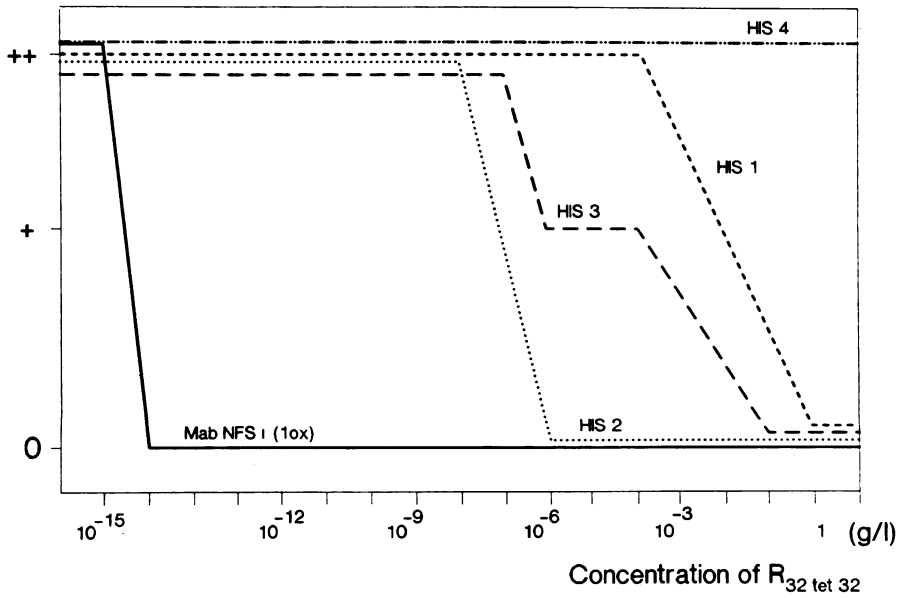

FIG. 2. IFA antigen competition assay. The surface reactivities of MoMAbs or serum samples at a 10-fold concentration of the endpoint titer were determined following incubation with decreasing concentrations of the recombinant R32tet32 antigen.

TABLE 1. Results of IFA antigen competition and ELISA[a]

Serum source or MoMAb (no. tested)	Mean IFA titer	No. of serum samples inhibited by peptide at concn[b]						No. of serum samples reactive with R32 in ELISA
		R32			$(NANP)_3$			
		10^{-1}	10^{-3}	10^{-6}	10^{-1}	10^{-3}	10^{-6}	
High-transmission areas								
Gabon (2)	9,050	1	1	1	0	0	0	1
Upper Volta (6)	2,850	4	3	2	1	1	0	4
Congo I (5)	11,090	4	0	0	2	0	0	3
Congo II (5)	10,050	1	0	0	1	0	0	2
Low-transmission areas								
North Senegal (3)	200	1	1	0	1	0	0	2
Thailand (2)	200	0	0	0	0	0	0	0
French Guyana (4)	1,600	3	1	0	1	0	0	2
MoMAbs								
NFS1 (1)	8×10^6	1	1	1	1	1	1	1
NFS2 (1)	1×10^6	1	1	0±	1	1	0±	1
Mice immunized with R32 (3)	17,200	3	3	3	ND	ND	ND	3

[a] Results are expressed as the number of serum samples whose IFA reactivity with the sporozoite surface could be inhibited by a given concentration of each peptide (concentrations expressed in grams per liter) and the number of serum samples reactive with the recombinant peptide R32 in ELISA (optical density > mean + 3 standard deviations of 14 control serum samples). In IFA competition assays, all serum samples were tested at a concentration of 10× the endpoint titer. Results obtained with a 16-mer synthetic peptide, $(NANP)_4$, were identical to those obtained with the 12-mer $(NANP)_3$ and are not shown. In the ELISA, all serum samples were tested at a 1/100 dilution.

[b] ND, Not done. 0±, Almost negative.

by the dot ELISA (data not shown). At a dilution of each serum corresponding to 10× the serum endpoint titer (thus giving a similar degree of IFA reactivity with the sporozoite surface), none of the 27 serum samples reacted with R32 bound to nitrocellulose, while under the same conditions, both MoMAbs, NFS1 and NFS2, and sera of mice immunized with R32 were strongly reactive. Increasing the concentration of the human sera to 100× the endpoint titer resulted in very faintly positive reactions in 12 of the 27 serum samples tested.

In all but two cases, the conclusions drawn from the two types of ELISAs performed and from the competition IFATs were in agreement, i.e., sera reacting directly with R32 in the ELISA could be inhibited in the IFAT by R32, and vice versa (nonrecognition in the first corresponding to absence of inhibition in the second). In agreement with this finding, results from the wet IFAT and those from the ELISA with R32 (Fig. 3) were poorly correlated ($r = 0.24$, $P > 0.1$); in contrast, a close correlation for serum samples from R32-immunized mice was found in the two assays (IFA titer, 1×10^4 to 3×10^4; ELISA ratio, 8 to 17), as well as for MoMAbs.

No relationship seemed to exist between the geographic origin of the serum donors, the degree of their exposure to sporozoites, and the results of immunoassays with the peptides (Table 1). Sera that were weakly reactive or nonreactive with NANP were found as frequently in subjects with high antibody titers from areas with high levels of malaria transmission by mosquitoes as they were in subjects with low antibody titers from low-transmission areas.

Finally, the prevalence of antibodies directed to CS epitopes outside the repeat region appeared to be low. By the ELISA with the CS repeatless recombinant protein, only 1 of the 27 individuals tested was clearly positive (ELISA ratio, 7.8), and another one was borderline (ratio, 1.5) (data not shown).

We next performed competition assays between MoMAbs and human sera in order to evaluate the occurrence in infected individuals of antibody specificities similar to those of the MoMAbs. Preincubation of sporozoites with high (100×) human antibody concentrations could inhibit further binding of MoMAb NFS2 (14 of 16 serum samples) to the sporozoite surface but was less effective with MoMAb NFS1 (inhibition occurred with 3 of 16 samples). These results were obtained only by using the human serum first and at a high concentration (100× was effective, but 10× was not) and using the MoMAb at a low concentration (2×).

In contrast, reverse experiments, in which sporozoites were first incubated with either or both of the MoMAbs, showed that no inhibition of the binding of any of the five human serum samples could be obtained. This was true over a wide range of concentrations of the two reagents, even

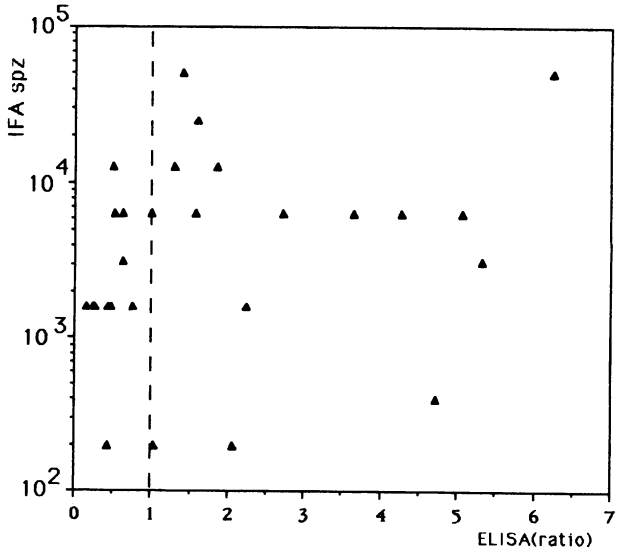

FIG. 3. Comparison of results from IFAT (wet sporozoite assay [IFA spz]) and ELISA (R32tet32 antigen).

TABLE 2. Surface IFA reactivity of HuMAbs and human sera with wet *P. falciparum* sporozoites

MAb or serum	% FITC-labeled sporozoites[a]										
	NF54	3D7	Th 1	Th 2	Th 4	Th 5	Th 6	Th 7	Th 8	Th 9	Th 10
HuMAbs											
1	75	0		68		30	18	5	0	20	0
2	14		66	7	0	0					
3	36	0	24	35	0	36	50	0	**13**	0	0
4	64	0	0	0	28	60	55	**100**	0	0	0
6	88	0					50	0	0	0	
7	57	0					19	0	0	54	**66**
11		**100**					80	0	0	0	0
12		0					88	0	0	0	0
Human sera											
HIS_1[b]	100		100	33	76	13	94	90	71	87	89
HIS_2[b]	100		100	62	80	32	90	62	40	72	69
E_3[c]	20	100	7	42	13	7	88	77	61	89	100
E_{59}[c]	15	100		22	0	0	85	9	**100**	100	85

[a] NF54 is a strain maintained in culture, and 3D7 is a clone from it, while Th 1 to Th 10 are sporozoites raised in mosquitoes fed on gametocytes from different patients from Thailand. FITC, Fluorescein isothiocyanate. Sample size ranged from 51 to more than 500. Values in boldface type indicate notable results.
[b] High-transmission area. HIS, Human immune serum.
[c] Low-transmission area.

when the MoMAbs were used at high concentrations (100× the endpoint titer) and the human sera were used at the extinction dilution (1×). MoMAbs did not prevent human immune sera from reacting with sporozoites (0 of 16 samples with strain NF54 and 0 of 5 samples with clone 7G8).

Antibody competition assays by ELISA led to the same conclusion, as the human antibodies were unable to block the binding of the two MoMAbs to the R32 antigen (data not shown).

These results indicate that human sera may contain antibody specificities similar to those of the MoMAbs, albeit at low levels, and that other antibody specificities differing from those of the MoMAbs are frequently prevalent at high titers. In turn, this suggests that additional epitopes which differ from those defined by the MoMAbs are accessible to antibodies on the surfaces of sporozoites.

To confirm that several distinct sporozoite antigens were defined by human antibodies, we decided to produce monoclonal antibodies of human origin. Sporozoite-reactive HuMAbs were selected by preliminary screening using the NF54 strain of parasites; therefore, the antibody specificities selected reflected antigens expressed on sporozoites from that strain. The reactivity of the HuMAbs was then studied with sporozoites from several isolates. A variable proportion of sporozoites from the NF54 line was labeled by any single HuMAb tested (Table 2). In contrast, sporozoites from 3D7, a parasite clone which derives from NF54, were recognized by only one HuMAb, which identified 100% of the cloned parasites. When reacted with Thai sporozoites, some HuMAbs recognized almost all sporozoites from one isolate but only some or none of the sporozoites from others (e.g., HuMAb 2 and sporozoites Th 1, 2, and 4 or HuMAb 4 and sporozoites Th 1, 4, and 7; Table 2). Two Thai isolates were nearly entirely defined by only one HuMAb (Th 7 by HuMAb 4 and Th 10 by HuMAb 7), and in this respect, results seem more or less similar to those obtained with sporozoites of the cloned line 3D7 in that the surface antigens appear to be homogeneous from one parasite to the next in these two isolates. On the basis of their reactivity with Thai sporozoites, HuMAbs 11 and 12 appear to have the same specificity; however, they yielded opposite results on 3D7 parasites, demonstrating that the epitope corresponding to that recognized by HuMAb 12 is absent from the 3D7 sporozoite surface. Other HuMAbs yielded intermediate results, recognizing variable percentages of sporozoites from nearly all isolates tested. In contrast, all sporozoites were labeled by the murine MAbs NFS1 and NFS2 used at a $1/10^6$ dilution, demonstrating the presence of the corresponding epitopes in the CS protein from each isolate (data not shown). The reproducibility of the results obtained by using HuMAbs was satisfactory in four separate experiments with NF54 parasites and in three experiments with 3D7 parasites (standard deviation, <20%).

Even when polyclonal antibodies were used, the same difference in the antigenic makeup of the parasites could be observed. In Table 2, the results were obtained by using four serum samples adjusted to two times their endpoint titers. Most sporozoites in most samples were recognized by serum samples from two adults living in a hyperendemic area who were exposed to large numbers of sporozoites and who had high titers of antisporozoite antibodies with a presumably wide range of specificities. In contrast, larger differences in sporozoite recognition from one sample to the other were found with two serum samples from children (3 and 4 years old) living in an area of low endemicity. These children, who had obviously been exposed to sporozoites less than the adults had been, had low antisporozoite antibody titers, and their sera presumably contained only a limited repertoire of antibody specificities.

Altogether, the above results indicate that a rather wide range of epitopes is expressed on the surface of *P. falciparum* sporozoites. They suggest that the expression of these antigens, which can be defined by human antibodies, may vary not only from one isolate to the next but also between sporozoites from a single isolate.

DISCUSSION

On the basis of the protection achieved by immunization with X-irradiated sporozoites and of the biological effect of MAbs directed to the repeat part of CS molecules of various malaria species, the CS group of molecules has been thought to be the protective antigen on sporozoites (11). However, results from recent vaccination experiments with the repeats from *P. falciparum* CS protein (1, 9) and T-cell epitope mapping studies (8) have revealed the existence of important limitations in its immunogenicity.

In our study, we have assessed by several means the correspondence of specificities of antibodies produced by individuals exposed to malaria to determinants on the artificial-vaccine candidates and on the native sporozoite surface antigens. Although the specific interpretation of each assay performed might appear debatable, taken together, the results obtained do not support previous reports suggesting that only one antigen defines the surface of *P. falciparum* sporozoites (11) and that a single region, that containing the tetrapeptide repeats, is immunodominant. On the contrary, our results indicate that epitopes contained within the CS protein are inconsistently and rather moderately immunogenic in humans and provide evidence in favor of the existence of additional sporozoite surface antigens, which account for a significant proportion of the antibody specificities developed by the human immune system.

The sera we studied were randomly selected from various parts of the world so as to reflect responses to a variety of parasite isolates and to represent different degrees of natural exposure. Antibodies to sporozoite surface antigens were detectable by IFATs in each of these individuals, and at very high titers in those exposed frequently to infectious mosquito bites (some of them on a daily basis). In contrast, in only some of the subjects studied were antibodies to the CS protein detected, either by ELISA with both the repeated and nonrepeated parts of the CS protein as antigens or by Western blotting with the extracted protein. Compared with results of other epidemiological studies with $(NANP)_x$ antigens, the prevalence of antibodies we found in the relatively small series studied is lower than that found in initial surveys (4) but comparable to that found in more recent ones (12).

Nonetheless, results from antibody competition assays clearly indicate that many of the serum samples studied did contain at least some antibodies with a specificity resembling that of one of the MoMAbs (NFS2) directed against the CS protein repeats. However, the discrepancy of results between IFAT and ELISA and the results from antigen competition assays suggest that in most instances, the prevalence and titers of antibodies to the CS protein, particularly the NANP repeats, are markedly lower than those directed to other surface epitopes. The results obtained with independent assays are confirmatory. They show a clear discrepancy between reactivity with native molecules on whole sporozoites and reactivity with artificial ones, particularly those patterned after what was thought to be the dominant antigen (for instance, five of the six serum samples with IFA titers greater than 10^4 had low or negative values by ELISA with the artificial peptides). In contrast, serum samples from mice immunized with the recombinant molecule R32, which served as controls, showed the same level of reactivity with both sporozoite and artificial molecules.

Thus, the immune response to the CS protein appears, under natural immunizing conditions, to be low in the exposed population and, moreover, varies from one individual to the other. The immune response does not appear to be related to the size of the parasite inoculum, supporting the existence of genetic restriction, which was recently reported for animals (7) and for humans (8). Clearly, this does not preclude the possibility that artificial priming with peptides from the CS molecule may result in the appropriate expansion of T and B cells. Although vaccination of volunteers with subunit vaccines consisting of either R32tet32 (1) or $(NANP)_3$ (9) may not have been performed under optimal conditions, the low and variable antibody responses obtained are in keeping with our observations of individuals immunized by the native CS molecule under field conditions. The differences we found in the concentrations of recombinant and synthetic peptides required to compete successfully in the assays may possibly be related to the smaller size of the synthetic peptide but more likely indicate that some antibodies are directed to epitopes which include the variant NVDP tetrapeptide, which is present in the recombinant R32 molecule but absent in the synthetic ones.

To date, the CS protein has been the only protein characterized on the surface of sporozoites following its identification by means of MoMAbs. The results of the IFAT, ELISA, and Western blotting suggested that humans develop antibodies that are frequently directed to surface epitopes not present in the CS molecule; this observation is supported by results obtained with human antibodies and a panel of sporozoites from different isolates. Some of the discrepancies observed between ELISAs and IFATs could be attributable to conformational differences between artificial and native molecules. However, results obtained with HuMAbs by the IFAT with molecules on intact sporozoites do not support this interpretation. The pattern of positivity given by the HuMAbs is in clear contrast with that given by antirepeat MoMAbs and is suggestive of the existence of a wide range of inconsistently expressed antigens. The same holds true for the pattern given by whole human sera obtained with the same isolates. This, in turn, may reflect the fact that there is a large diversity in the specificity of the dominant antibody in those sera.

Our study does not provide a characterization of the epitopes recognized by monoclonal and polyclonal human antibodies. In only two individuals were antibodies to an epitope located in the CS protein outside the repeat region detected. The others apparently corresponded to numerous epitopes whose expression may differ between clones constituting a single isolate and also between isolates. The fact that 100% of sporozoites from the parasite clone 3D7 were labeled by a single HuMAb is informative in this respect and permits an overall interpretation of the dissociated results found with uncloned isolates. The main characteristic of these antigens, compared with those antigens defined by MoMAbs, is that they are defined by the immune response of humans exposed to the parasite, and as such, they should be considered of interest.

Our conclusion that several antigens are accessible on the surface of *P. falciparum* sporozoites is consistent with recent experimental evidence in favor of the existence of non-CS protein surface molecules on *Plasmodium yoelii* sporozoites with antigenic features (3) and with gene structures (15) distinct from that of the gene encoding *P. yoelii* CS protein. However, from a practical point of view, it is not encouraging that no single HuMAb was able to label all the sporozoites studied. This suggests that none of the specific antibodies encountered in this study can be considered as a promising probe to define an alternative single vaccine candidate against *P. falciparum* sporozoites.

It can be concluded from the results presented above that a full analysis of the antigenic repertoire of *P. falciparum* sporozoites of various origins, including identification of the immunogens most consistently found in humans, is needed.

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