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Longitudinal Study of *Plasmodium falciparum* Polymorphic Antigens in a Malaria-Endemic Population

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Plasmodium falciparum **merozoite surface antigens MSP1 and MSP2 and an exported antigen, Exp-1, exhibit allelic polymorphism in natural populations. To explain this, one hypothesis is that antigen polymorphisms are maintained by frequency-dependent immune selection. An expectation of the hypothesis is that rare variants have an advantage over common variants because of a lower level of acquired immunity against them and thus increase in frequency until an equilibrium is attained. To test this hypothesis, the frequencies of polymorphic epitopes of MSP1, MSP2, and Exp-1 were determined among isolates from malaria patients in an urban area of The Gambia, during different periods of one transmission season (1988) and in different years (1982, 1983, 1988, and 1989). The frequencies remained very stable throughout the period of study, alternative epitope variants remaining either rare or common, without shifts in relative frequencies. These results are discussed with reference to the immune-selection hypothesis, with the conclusion that frequencies of the major dimorphic serological classes of MSP1 are probably not maintained by immune selection.**

The existence of antigenic diversity among "strains" of *Plasmodium falciparum* was suggested originally to explain the low rate of acquisition of immunity to malaria by inhabitants of endemic areas and the apparent recurrence of susceptibility among "immune" adults who travelled from one endemic area to another. In experimental infections of nonhuman primates, acquired immunity appeared to involve a "strain-specific" component (3, 33, 43), lending support to the importance of antigenic diversity. However, data from human infections were less consistent in this respect (1, 2, 22, 23).

Serological characterization of *P. falciparum* isolates subsequently demonstrated heterogeneity of soluble parasite antigens (44), internal and surface antigens (10, 27, 28), and infected erythrocyte surface components (20, 25, 42).

DNA sequencing has elucidated the genetic basis for the serological polymorphism of certain proteins, including the precursor to the major merozoite surface antigens (PMMSA or MSP1) (9, 19, 32, 39), a second merozoite surface antigen (MSA-2 or MSP2) (10, 34, 37, 40), and an exported protein (Exp-1) (36). MSP1 and MSP2 are among antigens considered for the development of a malaria vaccine. Immunization with MSP1 elicited a degree of immunity in monkeys (18, 31, 35). Immunization experiments with MSP2 have not been reported, but monoclonal antibodies (MAbs) against MSP2 partially inhibit in vitro invasion of erythrocytes (5, 34). Exp-1 may also be immunologically important, since it cross-reacts with the NANP(n) repeat epitope of the circumsporozoite protein (36).

It is not known whether polymorphisms in any of these antigens enable the parasite to evade variant-specific acquired immunity. If so, such polymorphisms might be subject to frequency-dependent selection, rare variants having a selective advantage because of lower levels of acquired immunity against them.

In this study, frequencies of variant epitopes on different domains of MSP1, MSP2, and Exp-1 have been determined for *P. falciparum* isolates collected from a small area of The Gambia over the period 1982 to 1989. The results are discussed with reference to the hypothesis of frequency-dependent selection by variant-specific immunity.

MATERIALS AND METHODS

Study area and patients. In The Gambia, malaria transmission is seasonal, with most infections with *P. falciparum* occurring from July through November (16). Blood samples were obtained from 424 malaria patients with *P. falciparum* infection at a density of ≥5 parasites per high-power field (×1,000 magnification), equivalent to a parasite density of ≥2,500 per μl (15). None of the patients was positive for other *Plasmodium* species. The patients presented to the Outpatients Departments of the Medical Research Council (MRC), Fajara, or the Royal Victoria Hospital, Banjul, from October through December 1982 (n = 46), October through December 1983 (n = 31), July through December 1988 (n = 228), and October through December 1989 (n = 119), and all lived within a radius of 7 km in an urban/peri-urban area described previously (7, 8).

Blood collection and parasite culture. A 0.3-ml sample of blood was obtained from each patient by finger prick or as part of a venous sample obtained for other studies, after consent, with approval from the Gambian government and MRC Scientific and Ethical Committees. The heparinized blood was washed three times in sterile phosphate-buffered saline, pH 7.3 (PBS), and parasites were cultured (41) for 24 to 48 h, until schizonts were obtained (29). After the cells were washed three times and resuspended at approximately 3% hematocrit in PBS, multispot schizont slides were prepared with approximately 20 μl of cell suspension per spot. The slides were dried in a well-air-conditioned room and stored under desiccation at −20°C.

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TABLE 1. Summary of MAbs specific for epitopes of MSP1, MSP2, and Exp-1 antigens of *P. falciparum*[a]

Antigen	Antibody	Reciprocal dilution for IFA[b]	Isotype	Epitope and location	References
MSP1	9.8-4-4-1	1,000	IgG1[c]	Conserved conformational	26, 27
	12.4-3-4	500	IgG1	Conserved conformational	26, 27
	12.8-2	1,000	IgG2b	Conserved domain 16–17, 16K fragment	13, 26
	12.2-1-1	2,000	IgG1	Polymorphic domain 2 repeats	26, 27, 29
	12 3D3.10	1,000	IgG2b	Polymorphic domain 2 repeats	24
	9.5-1-5-1	500	IgG2b	Polymorphic domain 3, 80K fragment	26, 27, 29
	13.2-3	2,000	IgG1	Polymorphic domain 3, 80K fragment	26
	12.1-5-4	2,000	IgG1	Polymorphic domain 4, 80K fragment	9, 13, 26, 27
	10-2B	2,000	IgG2a	Polymorphic domain 4, 80K fragment	9, 21
	9.2-6-2	2,000	IgG1	Dimorphic conformational*	13, 26, 27
	9.7-1	500	IgG1	Dimorphic conformational*	27, 29
	10.3-2	500	IgG1	Dimorphic conformational*	27, 29
	1-1C	500	IgG1	Dimorphic, 80K fragment†	21
	7.3-7	1,000	IgG2a	Dimorphic conformational†	26, 27, 29
	34-5	—[d]	IgG1	Dimorphic, 36K fragment†	14
	6.1-1-3	500	IgG1	Dimorphic domain 16, 40K fragment†	13, 26, 27, 29
	13.1-2	2,000	IgG1	Dimorphic domain 16, 40K fragment†	13, 26, 27, 29
	17.1-3	2,000	IgG1	Dimorphic domain 16, 40K fragment†	13, 26, 27, 29
	111.4	1,000	IgG1	Dimorphic domain 16–17, 16K fragment	19
MSP2	12.3-2-2	500	IgG1	Dimorphic group A-specific region‡	10, 29
	12.5-1-2	500	IgG1	Dimorphic group A-specific region‡	10, 29
	13.4-2-1	500	IgG1	Group A polymorphic repeat $(GSAG)_n$	5, 10
	8-5D	200	IgM	Polymorphic§	10, 38
	4-4F	200	IgM	Polymorphic§	10, 38
	8G10/48	300	IgG2b	Dimorphic group B-specific region (STNS)	10, 34
	8F6/49	50	IgG3	Group B polymorphic (DTPTATE)	10, 34
Exp-1	5.1-4	500	IgG1	Dimorphic NADP	36

[a] In this study, serological epitopes are referred to by the same code numbers as the MAbs which identify them. Conserved MSP1 epitopes were detectable on schizonts of all isolates. Polymorphic epitopes marked by identical symbols (*, †, ‡, or §) exhibit identical allelic distributions. Locations of MSP1 epitopes are shown schematically in Fig. 1.
[b] IFA, immunofluorescence assay.
[c] IgG1, immunoglobulin G1.
[d] —, undiluted.

MAbs. The working dilution, source, and specificity of each of the 27 murine MAbs against MSP1, MSP2, and Exp-1 are listed in Table 1. Three of the MAbs against MSP1 recognize epitopes which are conserved among all isolates previously studied, and 16 recognize MSP1 variant epitopes. Locations of the variant epitopes on different domains of MSP1 are shown schematically in Fig. 1. MAbs against MSP2 and Exp-1 all recognize variant epitopes.

Indirect immunofluorescence assay. Indirect immunofluorescence typing of each *P. falciparum* isolate was performed with individual MAbs on separate wells of acetone-fixed schizont slides (7, 29). A working dilution of each MAb (Table 1) was incubated on schizonts for ≥30 min. After careful removal of MAbs by Pasteur pipette, the slides were washed three times (1, 5, and 5 min) in PBS and dried. A 20-μl volume of a 1:100 dilution (in PBS, 1% bovine serum albumin, and 0.01% sodium azide) of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin antibody (ICN Immunobiologicals) was added to each spot, and the slides were incubated for ≥30 min. After two washes (1 and 5 min) in PBS, slides were stained with DAPI (4′,6-diamidino-2-phenylindole [Sigma Ltd.]; 10^{-6} [wt/vol] in PBS) for 1 min and again washed twice (1 and 5 min in PBS). The slides were dried and mounted under coverslips with Citifluor (Citifluor Ltd., City University, London) or glycerol-PBS (4:1).

Parasites were visualized by DNA-specific DAPI fluorescence (incident light of 390 to 440 nm) at a magnification of ×360 or ×600. For each isolate, the percentage of schizonts giving MAb-specific positive FITC fluorescence (incident light of 450 to 490 nm) was recorded for each MAb. Many isolates contained two or more *P. falciparum* clones, as resolved by double-labelled immunofluorescence using different pairs of MAbs (7). In the analyses described below, isolates in which a majority of parasites expressed a given epitope are distinguished from those in which only a minority expressed the epitope.

RESULTS

Scoring of *P. falciparum* isolates for expression of antigen epitopes recognized by MAbs. At least 200 schizonts from each isolate were scored for reactions with each of 27 MAbs by indirect immunofluorescence. All mature schizonts in every isolate gave specific parasite surface fluorescence with control MAbs 9.8, 12.4, and 12.8 against conserved epitopes of MSP1. Each of the other MAbs identified some isolates within which all schizonts were positive and other isolates within which all schizonts were negative. Such apparently homogeneous isolates were thus scored as being either positive or negative for a given epitope. However, genetically heterogeneous multiple-clone *P. falciparum* infections are common in The Gambia (4, 7), and thus there were isolates within which only a proportion of total schizonts reacted positively with one or more of the MAbs against polymorphic epitopes. The statistical analyses considered

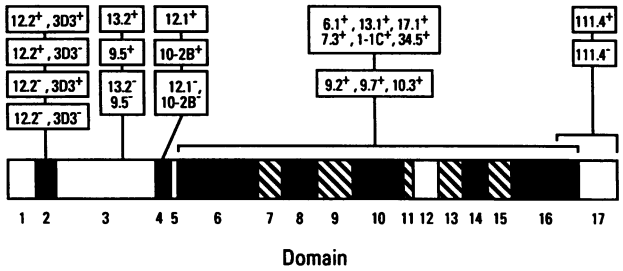

FIG. 1. A scheme of the locations of polymorphic epitopes at different domains of MSP1. The sequence of MSP1 is schematically divided into 17 domains or "blocks" (39) according to the proportion of conserved amino acid residues, as follows: conserved, unshaded; semiconserved, hatched; polymorphic, fully shaded. These are relative terms, and there are nonconserved residues even within "conserved" domains. The specificities of MAbs recognizing polymorphic epitopes are shown in the different boxes at the top of the figure. For example, on domain 16/17 there are two alternatives (positive or negative for MAb 111.4), while on domain 4 there are three alternatives (positive for MAb 12.1, MAb 10-2B, or neither). (Reprinted from *Experimental Parasitology* [9] with permission of the publisher.)

positive all isolates within which the majority (50 to 100%) of schizonts expressed a given epitope. Although not included within the statistical analyses, data on the percentages of isolates within which more than 1% of schizonts expressed a given epitope are also presented.

Comparison of polymorphic epitope frequencies during a single transmission season. Table 2 compares frequencies of different MSP1, MSP2, and Exp-1 epitopes among clinical isolates collected at the beginning (July and August), middle (September and October), and end (November and December) of the 1988 transmission season. The MSP2 epitope 12.3 occurred less frequently (and hence the alternative epitope 8G10/48 more frequently) during July and August than during September and October or November and December (chi-square, $P < 0.05$ for each comparison). There were no significant differences in frequencies of any other epitopes between the periods.

Comparison of polymorphic epitope frequencies during the period 1982 to 1989. To investigate whether a slow antigenic drift may occur over a period of several years, frequencies of polymorphic epitopes were recorded between 1982 and 1989. Figure 2 compares epitope frequencies among *P. falciparum* isolates collected in the same area as above during the October to December periods of 1982 ($n = 46$), 1983 ($n = 31$), 1988 ($n = 101$), and 1989 ($n = 119$). Only two statistically significant frequency changes were recorded. The epitope 12.2 on the repeat domain of MSP1 was more frequent in 1983 than in each other year (chi-square, $P < 0.05$ for each comparison), and the epitope 12.1 on domain 4 of MSP1 was more frequent in 1982 than in each other year (chi-square, $P < 0.05$ for each comparison).

Data presented in Table 2 and Fig. 2 illustrate a very striking difference between the frequencies of the two alternative major serological classes of MSP1, here identified by epitopes on domains 6 to 16 (Fig. 1 and Table 1). The frequency of the "MAD20-like" class (identified by epitopes 9.2, 9.7, and 10.3, with 9.2 shown as an example in Table 2 and Fig. 2) was high, >95%. In contrast, the frequency of the "K1/Wellcome-like" class (identified by epitopes 6.1, 7.3, 13.1, 17.1, 1-1C, and 34-5, with 6.1 shown as an example in Table 2 and Fig. 2) was less than 5%. This difference remained a stable characteristic of the parasite population over the period of the study.

TABLE 2. Frequencies of epitopes marking polymorphic domains of MSP1, MSP2, and Exp-1 at different periods of the 1988 transmission season[a]

Antigen	Domain	Epitope	Frequency (%)					
			July–Aug.		Sept.–Oct.		Nov.–Dec.	
			A	B	A	B	A	B
MSP1[b]	2	12.2	25.5	37.3	29.6	45.2	26.2	40.5
	2	123D3	27.5	31.4	20.0	28.9	11.9	26.2
	3	13.2-3	52.9	66.6	48.2	63.0	51.2	63.4
	3	9.5	39.2	45.1	38.5	55.0	43.9	53.7
	4	12.1	33.3	45.1	32.6	50.4	45.2	59.5
	4	10-2B	66.7	72.8	68.2	75.6	54.8	64.3
	6–16	6.1	2.0	2.0	5.2	5.2	2.4	2.4
	6–16	9.2	98.0	98.0	94.8	97.0	97.6	97.6
	16–17	111.4	45.1	58.8	41.5	53.4	52.4	64.3
MSP2[c]		12.3	<u>50.0</u>	62.0	69.2	80.4	78.1	90.3
		8G10/48	<u>50.0</u>	52.0	30.2	45.8	21.4	40.5
		13.4	14.0	16.0	8.3	13.6	7.1	14.3
		8-5D	46.0	64.0	54.6	66.7	61.0	70.8
		8F6/49	16.7	20.9	15.5	27.6	13.2	21.1
Exp-1[d]		5.1	30.6	51.0	30.1	45.9	28.6	50.0

[a] Columns A denote the percentages of isolates in which 50 to 100% of schizonts were positive for a given epitope; columns B denote the percentages in which 1 to 100% were positive. Comparing frequencies in columns A between periods, the two values which were significantly different from those in later periods (chi-square, $P < 0.05$) are underlined. Omitted from the table are MSP1 epitopes 7.3, 13.1, 17.1, 1-1C, and 34-5, which showed parasite distributions identical to that of epitope 6.1; MSP1 epitopes 9.7 and 10.3, which showed specificity identical to that of epitope 9.2; MSP2 epitope 12.5, with specificity identical to that of 12.3; and MSP2 epitope 4-4F, with specificity identical to that of 8-5D.

[b] Number of isolates: 51 for July and August, 135 for September and October, and 42 for November and December.

[c] Number of isolates: 50 for July and August, 133 for September and October, and 41 for November and December.

[d] Number of isolates: 49 for July and August, 133 for September and October, and 42 for November and December.

DISCUSSION

The main finding of the study was that the relative frequencies of MSP1, MSP2, and Exp-1 variant epitopes among *P. falciparum* isolates from Gambian patients remained stable during the period 1982 to 1989. Only the MSP1 epitopes 12.1 and 12.2 had significantly higher frequencies in 1982 and 1983, respectively, although these differences are no more than expected by chance (because of the large total number of chi-square comparisons made). The frequencies were also stable throughout a single transmission season (with only two significant differences among all the chi-square comparisons, as would again have been expected by chance).

Therefore, the more common variants remained at a higher frequency over several years, and the rare variants remained at a lower frequency. This was particularly notable with respect to the two alternative types of MSP1 domains 6 to 16. This "dimorphic" region of MSP1 extends over most of the sequence (32, 39) and is represented in the sequence divergence between laboratory isolates MAD20 and K1/Wellcome (39). All MAbs recognizing this dimorphic region have one of two alternative specificities and are useful

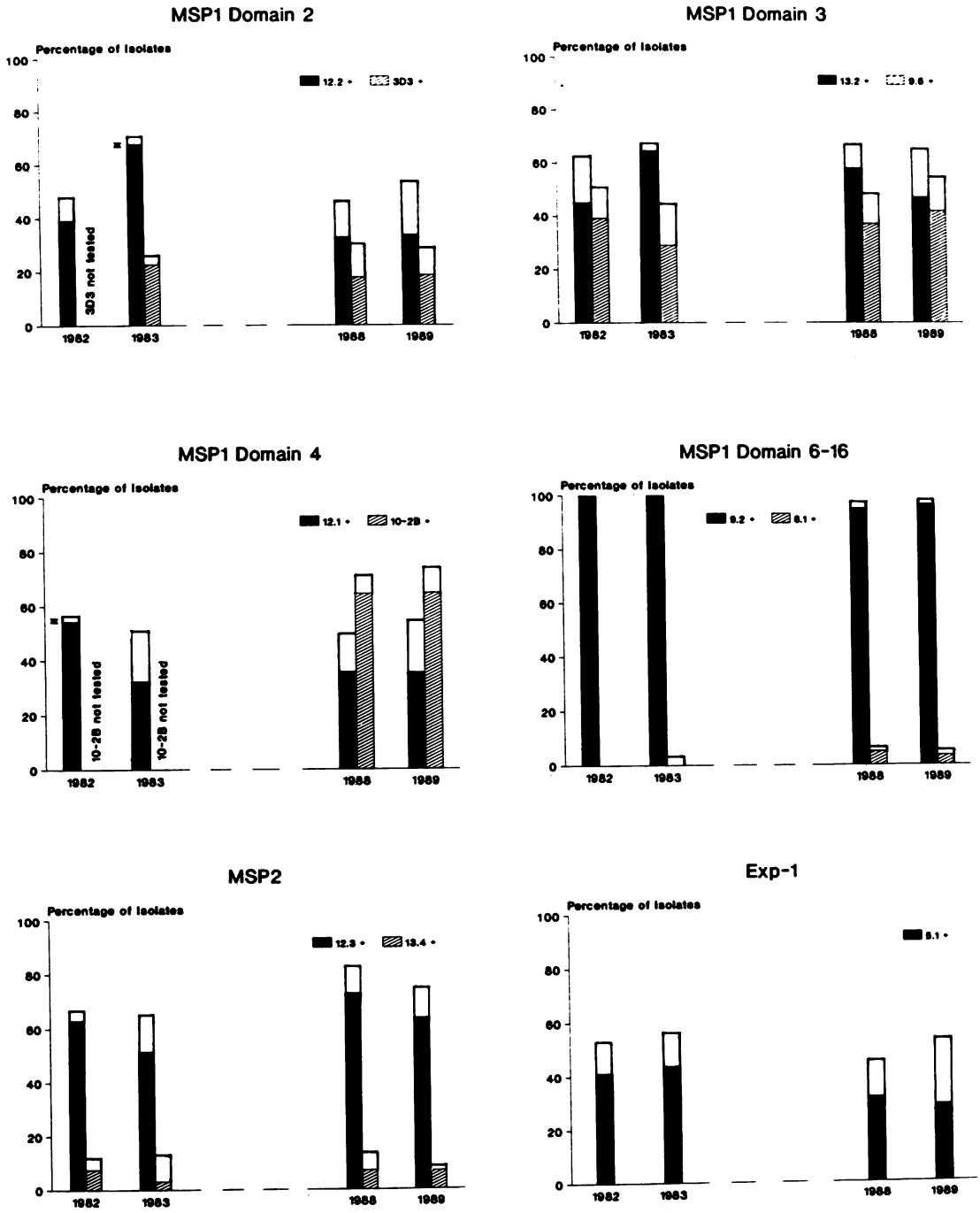

FIG. 2. Percentage of isolates containing parasites expressing different polymorphic epitopes of MSP1, MSP2, and Exp-1 from October through December 1982 (n = 46), 1983 (n = 31), 1988 (n = 101), and 1989 (n = 119). Shaded bars indicate the proportion of isolates in which 50 to 100% of parasites expressed a given epitope, and open bars show the proportion of isolates in which 1 to 100% of parasites had each epitope. Asterisks mark the two values which were higher in one year (chi-square, $P < 0.05$). Omitted from the figure are MSP1 epitopes 7.3, 13.1, 17.1, 1-1C, and 34-5, which showed parasite distribution identical to that of epitope 6.1 (epitopes 1-1C and 34-5 were tested for only in 1988 and 1989 isolates); MSP1 epitopes 9.7 and 10.3, which showed specificity identical to that of the epitope 9.2; and MSP2 epitope 12.5, with specificity identical to that of 12.3.

markers of the dimorphism even though naturally acquired human antibodies may be directed against different epitopes. The K1/Wellcome-like type (here distinguished by epitopes 6.1, 7.3, 13.1, 17.1, 1-1C, and 34-5) remained at a very low frequency (<5%), while the MAD20-like type (distinguished by epitopes 9.2, 9.7, and 10.3) maintained a high frequency (>95%). During the intervening years 1984 to 1987, the population was not systematically sampled. However, in nine isolates available from 1986, all parasites had the common MAD20-like dimorphic type, confirming that there

was no significant frequency shift during this time (unpublished data; isolates supplied by M. J. Blackman).

Theoretically, a frequency-dependent selection can maintain genetic polymorphisms with either stable or fluctuating variant frequencies, depending on whether equilibrium frequencies have been or are being reached (6, 17). If acquired variant-specific immunity operated on the dimorphic regions of MSP1, it is difficult to explain how the two alternative types could remain at such greatly differing frequencies at equilibrium. Moreover, it is improbable that the parasite population would maintain what could be described as a "conserved dimorphism" in the allelic sequences in response to a specific immune response. Mechanisms other than frequency-dependent selection by acquired variant-specific immunity are therefore required to explain the sequence dimorphism at domains 6 to 16 in MSP1. Since MSP1 may be a parasite receptor involved in the recognition of the host erythrocyte (30), one such mechanism may be a corresponding dimorphism in a ligand of the erythrocyte surface in the human population.

However, more extensive polymorphisms exist in domains of tandem repeats in MSP1 (32, 39) and MSP2 (10, 37, 40), and these might be subject to immune selection. In a hyperendemic area, such as The Gambia, it may be that equilibrium frequencies of such variants have been reached, which could explain the stable frequencies of, e.g., epitopes 12.2 and 3D3 on MSP1 and 13.4 on MSP2. Alternatively, the polymorphisms may be neutral with respect to immunity, and the lack of change in these frequencies may simply result from an absence of selection.

Temporal variations in the prevalence of an S-antigen epitope were recorded in villages of Papua New Guinea (11). It was argued that these variations could possibly result from frequency-dependent immune selection (12). However, as pointed out elsewhere (8), random fluctuations in allelic frequencies are likely to occur in *P. falciparum* populations in villages because of genetic drift in small populations, so selective mechanisms are not required to explain the data.

In the present study, the frequencies of each epitope have been analyzed separately, independently of one another. However, different epitopes on the same protein are likely to be strongly associated genetically or structurally. For example, even though intragenic recombinations occur in the MSP1 gene (9, 32, 39), strong associations between variant epitopes located on different domains of the protein exist in local populations (9). Therefore, a consideration of allelic serotype frequencies, in addition to individual variant epitope frequencies, is necessary. In The Gambia, MSP1 and MSP2 allelic serotype frequencies in 1988 and 1989 were resolved, and they remained similar in both years (8).

In conclusion, the present results suggest that some major antigen polymorphisms, particularly the dimorphism which includes most of the sequence of MSP1, are not maintained by frequency-dependent immune selection. It is not excluded that other polymorphisms in MSP1 and MSP2 might be balanced by effects of frequency-dependent immune selection, although immunological studies are now required to demonstrate such variant-specific immune responses.

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