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Natural Human Antibody Responses to *Plasmodium vivax* Apical Membrane Antigen 1 under Low Transmission and Unstable Malaria Conditions in Sri Lanka

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***Plasmodium vivax* apical membrane antigen 1, an important malaria vaccine candidate, was immunogenic during natural malaria infections in Sri Lanka, where low transmission and unstable malaria conditions prevail. Antibody prevalence increased with exposure in areas where malaria was or was not endemic. A marked isotype switch to cytophilic (immunoglobulin G1 [IgG1]/IgG3) antibodies was evident with increasing exposure exclusively in residents from areas of endemicity.**

Asexual blood stages of the *Plasmodium* life cycle are responsible for the acute symptoms of malaria. Immunity to asexual blood stages in the infected host is important in reducing the clinical burden and in eliminating the parasite load. Therefore, molecules involved in erythrocyte invasion by *Plasmodium* merozoites are attractive targets for effective immune intervention since antibodies to merozoite surface proteins have been shown to block adhesion and invasion of host erythrocytes (3, 4, 12). Apical membrane antigen 1 (AMA-1) is a leading vaccine candidate for *Plasmodium* and is ubiquitously present in all *Plasmodium* species (18). A high prevalence of natural antibodies to *P. falciparum* AMA-1 has been demonstrated in populations with lifelong exposure to malaria (10, 14, 16), but such information is only beginning to appear for *P. vivax* AMA-1 (13). The lack of data for this important human pathogen prompted the present cross-sectional study which for the first time examined the nature of the *P. vivax* AMA-1 antibody response during acute *P. vivax* infections in populations of Sri Lanka that are endemic and nonendemic for malaria.

Following ethical approval by the ethics review committee of the University of Colombo, Sri Lanka, blood samples were collected with informed consent from *P. vivax*-infected patients (age, >15 years) from Anuradhapura (8°22′N, 80°20′E; n = 84), Kataragama (6°25′N, 81°20′E; n = 111), and Colombo (7°55′N, 79°50′E; n = 94) during 1999 and 2000. Healthy individuals with no history of malaria from Colombo served as controls (n = 30). Anuradhapura and Kataragama are predominantly areas of *P. vivax* malaria endemicity with low transmission and unstable malaria conditions (entomologic inoculation rates for *Anopheles* species are one and four infectious bites per person per year for *P. vivax* and *P. falciparum*, respectively) (11). During 1995 to 2000, the annual parasite incidence due to *P. vivax* was 80 to 160 and 40 to 5 per 1,000 individuals in Kataragama and Anuradhapura, respectively (2). The corresponding figures for *P. falciparum* were 10 to 20 and 40 to 5, respectively (2). The majority of patients from Colombo, which is malaria free (2, 6), were adults returning from visits to regions with *P. vivax* transmission. The test and control groups were comparable in age (mean, 30 years) (analysis of variance [ANOVA], $P > 0.05$) and gender (chi-square test, $P > 0.05$). Residents from Kataragama showed a significantly higher number of previous malaria infections (median, 6) than did residents from Anuradhapura (median, 2) and Colombo (median, 1) (Mann-Whitney U test, $P < 0.001$). Further, patients from Colombo manifested significantly higher parasite densities (median, 0.08) than did patients from Kataragama (median, 0.05) and Anuradhapura (median, 0.04) (Mann-Whitney U test, $P < 0.05$).

The total (immunoglobulin M [IgM] plus IgG) and isotype-specific anti-*P. vivax* AMA-1 antibodies in the acute-phase sera were assayed against recombinant protein *PV* 66/AMA-1 (9) by indirect microplate assay (15) and antibody sandwich enzyme-

TABLE 1. Prevalence, magnitude, and reciprocal endpoint titers of anti-AMA-1 total antibodies (IgM plus IgG) of acute-phase *P. vivax* patients from the three study areas

Area	No. of samples	Prevalence (%)	Magnitude (OD)		Reciprocal endpoint titer		
			Mean	SEM	Median	Percentile	
						25%	75%
Anuradhapura	84	47	1.063	0.058	900	200	51,200
Kataragama	111	54	1.003	0.043	200	100	6,400
Colombo, total	94	50	1.422	0.054	25,600	1,600	51,200
Colombo, PNE	40	30	1.404	0.121	25,600	0	2,500
Colombo, PE	51	65	1.398	0.061	25,600	0	51,200

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TABLE 2. Prevalence and magnitude of anti-AMA-1 IgM antibody response of acute-phase *P. vivax* patients from the three study areas

Area	No. of samples	Prevalence (%)	Magnitude (cutoff value, 0.304)	
			Mean	SEM[a]
Anuradhapura	40	56	0.582	0.08
Kataragama	45	35	0.622	0.056
Colombo, total	40	95	0.878	0.06
Colombo, PNE	11	91	1.102	0.146
Colombo, PE	29	97	0.768	0.058

[a] SEM, standard error of the mean.

linked immunosorbent assay (ELISA) (5), respectively. As the optical density (OD) values from the ELISA for the 30 normal controls were normally distributed and age matched with test serum samples, the cutoff value for this test was calculated to be the mean OD value plus two standard deviations for the normal controls. The mean OD at 405 nm obtained at serum dilutions of 1:100 and 1:10 was considered a measure of the magnitude of the anti-AMA-1 total and isotype-specific antibody responses of each individual, respectively. Mean OD values obtained for test samples falling over and above this cutoff level were expressed as positive responses, and these values were used to derive mean antibody magnitudes of each test area. Endpoint titers for total antibodies were determined using twofold serial serum dilutions starting from 1:100. The endpoint titer was the reciprocal of the highest test sample dilution that gave a reading above the cutoff provided by the appropriate dilution of the normal control. To adjust the affinity differences between the IgG isotype-specific monoclonal antibodies, the specific OD values were adjusted by calibrating the assay using a reference serum (human standard serum NOR-01; Nordic Immunology). The OD values obtained were compared with the actual values for the reference serum and used to calculate compensation factors for the different isotypes, which are the ratios of OD for the given isotype to that of IgG1 (17). The derived compensation factors for IgG1, IgG2, IgG3, and IgG4 were 1, 0.32, 0.82, and 0.68, respectively, and these were used to adjust the ELISA values.

Total antibody prevalence (percentage of antibody-positive sera) in all three test sites was around 50% (Table 1). Patients from Colombo with no previous exposure (PNE) to malaria showed significantly lower antibody prevalence (chi-square 8.13, $P < 0.01$) than that of their previously exposed (PE) counterparts from Colombo and the two regions of endemicity (chi square, 8.44; $P < 0.05$). Antibody magnitudes (ANOVA, $P < 0.001$) and endpoint titers (Kruskal-Wallis test, $P < 0.001$) were significantly higher in patients from Colombo than from areas of endemicity (Table 1). No significant difference ($P > 0.05$) was evident between the antibody magnitudes and endpoint titers of both PE and PNE responders to *P. vivax* AMA-1 from Colombo.

Positive samples were randomly assessed for IgM and the four IgG subclasses. The IgM prevalence (chi-square test, $P < 0.001$) and magnitude (ANOVA, $P < 0.01$) results in Colombo were significantly higher than those of residents from areas of endemicity (Table 2). Although higher IgM magnitudes were evident in PNE individuals than in the PE group in Colombo (t test, $P < 0.05$) (Table 2), their IgM prevalence results were similar (chi-square test, $P > 0.05$). The IgM prevalence (chi-square test, $P > 0.05$) and magnitude results (t test, $P > 0.05$) of the two areas of endemicity were comparable (Table 2). Significantly elevated IgG1 and IgG3 responses of individuals from all test areas were apparent relative to IgG2 and IgG4 responses (paired t test, $P < 0.001$) (Table 3).

As most malaria infections are symptomatic in patients from Sri Lanka (7, 11) and malaria episodes are routinely confirmed by microscopy prior to treatment, the self-reported number of past clinical episodes (*P. vivax* and *P. falciparum*) was used to classify these patients into four categories of cumulative exposure to malaria: (i) PNE, (ii) one to two previous infections, (iii) three to five previous infections, and (iv) more than five previous infections. A positive trend between antibody prevalence and previous exposure for groups of individuals was evident in all test sites but was statistically significant only in individuals from Colombo (chi square for linear trend, 10.4; $P = 0.001$).

Individuals in each test area were classified into two groups for the analysis of anti-*P. vivax* AMA-1 IgM, IgG1, and IgG3 variation associated with previous exposure to malaria: (i) those with IgM responses, with or without IgG1 and IgG3 [termed IgM (±IgG)], and (ii) those with IgG1 and IgG3 without IgM responses. Results from individuals of both areas of endemicity were pooled ($n = 61$) and compared with those from areas of nonendemicity ($n = 40$) (Fig. 1). The proportion of individuals from Colombo with IgM responses (±IgG) ($n = 38$), relative to those with IgG1 and IgG3 ($n = 2$) responses, was significantly higher than that for individuals from areas of endemicity ($n = 31$ and $n = 30$, respectively) (chi-square test, $P < 0.001$). The percentage of individuals with IgM (±IgG) from regions of endemicity was reduced with increasing exposure and was minimal in those with more than five past infections. In parallel, the percentage of individuals with IgG1 and IgG3 responses was maximal (75%) among those with more than five past malaria infections (chi square for linear trend, 10.75; $P = 0.001$) (Fig. 1A). However, a similar pattern of antibody shift towards IgG1 and IgG3 with a concurrent reduction of IgM with increasing exposure was not detectable for

TABLE 3. Prevalence and magnitude of anti-AMA-1 IgG subclasses of acute-phase *P. vivax* patients from the three study areas

IgG isotype (cutoff value)	Anuradhapura ($n = 40$)		Kataragama ($n = 45$)		Colombo ($n = 40$)	
	Prevalence (%)	Magnitude (SEM)	Prevalence (%)	Magnitude (SEM)	Prevalence (%)	Magnitude (SEM)
IgG1 (0.096)	75	0.65 (0.06)	72	0.74 (0.05)	89	0.86 (0.04)
IgG2 (0.027)	32	0.12 (0.02)	15	0.093 (0.006)	67	0.12 (0.006)
IgG3 (0.11)	88	0.456 (0.047)	54	0.361 (0.047)	85	0.411 (0.039)
IgG4 (0.077)	52	0.166 (0.035)	67	0.086 (0.01)	76	0.085 (0.004)

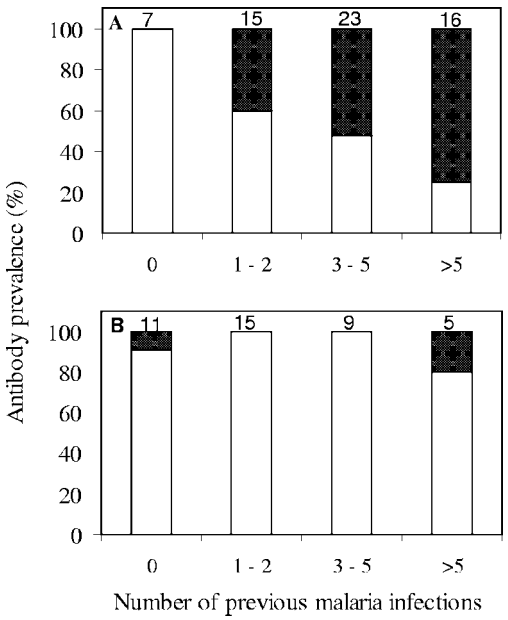

FIG. 1. Pattern of isotype-specific antibody prevalence of individuals from areas of malaria (A) endemicity and (B) nonendemicity. Individuals with IgM responses, with or without IgG1 and IgG3 (□), and individuals with IgG1 and IgG3 without IgM responses (■) to AMA-1 in groups of different past exposure. The number of individuals tested in each past exposure group is indicated above the bars.

P. vivax AMA-1 in residents from the area of nonendemicity (chi square for linear trend, 0.23; $P > 0.05$).

The observed anti-AMA-1 antibody prevalence indicates that irrespective of the degree of malaria transmission, AMA-1 is highly immunogenic during a *P. vivax* infection among populations from Sri Lanka. The anti-*P. vivax* AMA-1 antibody prevalence among PNE patients, though significantly lower than for PE patients from Colombo, suggests that *P. vivax* AMA-1 was immunogenic even in individuals with a limited antigenic exposure. The positive trend between responding proportions and previous exposure further illustrated this fact.

However, it is imperative to reason out the nonresponsiveness to PV66/AMA-1 of approximately 45% of residents from areas of endemicity. The prevailing low degree of malaria endemicity in Sri Lanka (11), the extent to which the recombinant construct based on the Sal I strain (9) used in this study represents the full repertoire of cross-reactive alleles of *P. vivax* AMA-1 (8) in the study areas, and genetically controlled mechanisms probably act as determinants of this observation.

In the present study, the reduction of IgM prevalence against increasing exposure to malaria among residents from areas of endemicity was evident with stabilized IgG1 and IgG3 prevalence, as has been reported for falciparum malaria (1). Thus, under low transmission, it was evident that with increasing exposure to malaria, the bias of the anti-*P. vivax* AMA-1 antibody response was towards cytophilic IgG1 and IgG3 isotypes, both at population and at individual levels. In contrast, this isotype switch was not apparent in individuals from the area of nonedemicity, where equally distributed prevalences of both IgG subclasses as well as IgM among differentially exposed patients were evident. The switch to IgG1 and IgG3 anti-*P. vivax* AMA-1 antibody responses in repeatedly and frequently exposed individuals from areas of endemicity in this study could point to a "protective" nature of these antibodies against *P. vivax* infections. In that case, the individuals in areas of endemicity could have had many more subclinical *P. vivax* infections than reported for the patients in Colombo. This could explain the observed IgG1- and IgG3-boosting effect in this population.

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