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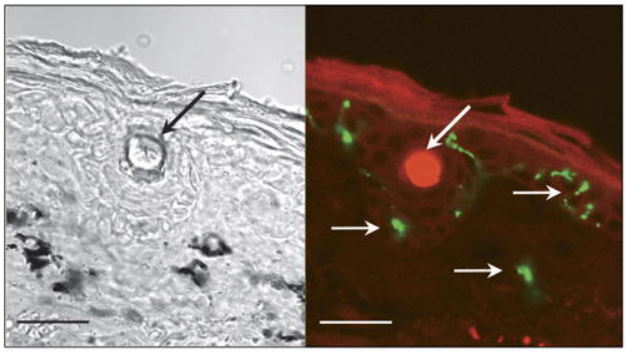

The frontispiece is from Kumkate et al., page 214

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Comparison of naturally acquired antibody responses against the C-terminal processing products of *Plasmodium vivax* Merozoite Surface Protein-1 under low transmission and unstable malaria conditions in Sri Lanka

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Abstract

We report here, for the first time, a comparison of naturally acquired antibody responses to the 42 and 19 kDa C-terminal processing products of *Plasmodium vivax* Merozoite Surface Protein-1 assayed by ELISA using p42 and p19 baculovirus-derived recombinant proteins, respectively. Test populations comprised patients with microscopy confirmed acute *P. vivax* infections from two regions endemic for vivax malaria where low transmission and unstable malaria conditions prevail, and a non-endemic urban area, in Sri Lanka. The antibody prevalence to the two proteins, both at the individual and population levels, tend to respond more to p42 than to p19 in all test areas, where >14% of individuals preferentially recognized p42, compared with <2% for p19. In patients with no previous exposure to malaria, 21% preferentially recognized p42, whereas none exclusively recognized p19. A significantly lower prevalence of anti-p19 IgM, but not anti-p42 IgM, was observed among residents from endemic areas compared with their non-endemic counterparts. Individuals from both endemic areas produced significantly less anti-p19 IgM compared with anti-p42 IgM. IgG1 was the predominant IgG isotype for both antigens in all individuals. With increasing exposure to malaria in both endemic areas, anti-p19 antibody responses were dominated by the functionally important IgG1 and IgG3 isotypes, with a concurrent reduction in IgM that was lacking in the non-endemic residents. This antibody switch was also reflected for PvAMA-1 as we previously reported with the identical battery of sera. In contrast, the antibody switch for p42 was restricted to endemic residents with more extensive exposure. These results suggest that an IgM-dominated antibody response against the p42 polymorphic region in endemic residents may interfere with the development of an IgG-dominated "protective" isotype shift to p19, that may complicate vaccine development.

Keywords: Sri Lanka; Low transmission; Unstable malaria; *Plasmodium vivax*; MSP-1 C-terminal fragments; Immuno-epidemiology; Antibody switch

1. Introduction

Plasmodium vivax is the most widely distributed human malaria parasite outside Sub-Saharan Africa accounting for 80–90 million cases per year globally and is the most prevalent among the four human malaria species in Asia

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and Latin America (Mendis et al., 2001). A protective *P. vivax* vaccine would have a considerable socio-economic impact on populations residing in endemic areas, especially in light of emerging drug resistance to this parasite (Dua et al., 1996).

The C-terminal region of the Merozoite Surface Protein-1 (MSP-1) is a leading malaria vaccine candidate for both *Plasmodium falciparum* and *P. vivax* malaria. The 42- and 19-kDa C-terminal fragments of PfMSP-1 have been validated as potential vaccine candidates by several efficacy trials in primate and rodent systems (Chang et al., 1996; Tian et al., 1997; Egan et al., 2000), and by in vitro invasion inhibition studies with monoclonal and polyclonal antibodies (Blackman et al., 1994; O'Donnell et al., 2001).

The study of antibody responses to potential asexual erythrocytic *Plasmodium* vaccine candidates in endemic sites can help in understanding natural protective immune mechanisms that may provide useful insights for vaccine development. In particular, anti-merozoite antibodies have been shown to be active in in vitro functional assays, indicating a role in protective efficacy (Bouharoun-Tayoun et al., 1995; Kumaratilake and Ferrante, 2000). Antibody responses to C-terminal PfMSP-1 (MSP-1p42 and MSP-1p19) are associated with a reduced risk of clinical malaria (Shai et al., 1995; Al-Yaman et al., 1996; Perraut et al., 2005). The prominence of IgG1 and IgG3 antibodies in the anti-PfMSP-1p19 IgG response (Egan et al., 1995), and their association with clinical immunity has been described (Shi et al., 1996).

Comparatively less is known about *P. vivax* MSP-1 (PvMSP-1), due in part to the lack of an in vitro culture system for this parasite. However, molecular and processing homologies to PfMSP-1 suggest it has similar function(s) (Galinski and Barnwell, 1996), and may be involved in *P. vivax* merozoites binding to reticulocytes (Rodriguez et al., 2002). PvMSP-1 was identified as a 200-kDa protein (Udagama et al., 1987; del Portillo et al., 1988) that was subsequently cloned and sequenced (del Portillo et al., 1991; Gibson et al., 1992). C-terminal PvMSP-1p42 and PvMSP-1p19 recombinant homologues of PfMSP-1p42 and PfMSP-1p19 were produced in the baculovirus expression system (Longacre et al., 1994). Importantly, baculovirus-expressed *Plasmodium cynomolgi* MSP-1 C-terminal p42 and p19 recombinant proteins (Longacre, 1995; Holm et al., 1997) conferred a high degree of protection against a challenge infection in the toque monkey, *Macaca sinica* (Perera et al., 1998). These results are particularly notable because this natural host-parasite system is highly analogous to *P. vivax* infection in humans. Immuno-epidemiological studies have shown a high prevalence of antibodies against C-terminal regions of PvMSP-1 and an increase of IgG prevalence with exposure to *P. vivax* malaria in endemic regions of South America (Soares et al., 1999).

As vaccine development is such a costly enterprise, early validation and optimization of the best candidates is a high priority. In this context, it was considered important to focus vaccine development efforts on one or the other of the PvMSP-1p42 or p19 antigens. Thus, the cross-sectional immuno-epidemiological study presented here was designed to compare total and isotype-specific natural antibody responses to PvMSP-1p42 and p19 in sera of individuals infected with *P. vivax* from malaria-endemic and non-endemic regions in Sri Lanka, where low transmission and unstable malaria conditions prevail (Rajendram and Jayewickreme, 1951; Mendis et al., 1990) and *P. vivax* currently accounts for 60–80% of all malaria infections (Ministry of Health, 2001). This study was carried out using PvMSP-1p42 (p42) and PvMSP-1p19 (p19) recombinant proteins expressed in the baculovirus system, which is considered to optimally reproduce the complex conformational structures and epitopes of the MSP-1 C-terminal antigens (Longacre et al., 1994; Chitarra et al., 1999; Pizarro et al., 2003). The results are discussed with regard to the optimization of PvMSP-1-based vaccine strategies.

2. Materials and methods

2.1. Study subjects

Age (>15 years) and gender matched patients with microscopy confirmed acute *P. vivax* infections from the General Hospital, Anuradhapura (8°22′N, 80°20′E), Malaria Research Station, Kataragama (6°25′N, 81°20′E) and the National Hospital, Colombo (7°55′N, 79°50′E), were included in the study (Wickramarachchi et al., 2006).

Anuradhapura and Kataragama are predominantly *P. vivax* malaria endemic areas situated in dry zone regions of the island (Mendis et al., 1990). From 1995 to 2000 the annual incidence of *P. vivax* ranged from 80 to 160, and 5 to 40 per 1,000 population in Kataragama and Anuradhapura, respectively, with a corresponding *P. falciparum* incidence of 10–20 and 5–40 (Briët et al., 2003). Since Colombo is situated in the wet zone and is malaria-free (Briët et al., 2003), most patients were infected during visits to *P. vivax* endemic dry regions (Fonseka and Mendis, 1987).

Where appropriate, comparisons were drawn between patients from Colombo with no previous exposure (PNE) to malaria and their previously exposed (PE) counterparts from Colombo and the two endemic areas.

2.2. Serum

All blood samples were obtained after informed consent, following approval by the University of Colombo Ethical Review Committee. Subjects included acute *P. vivax* patients presenting during 1999–2000 and healthy individuals from Colombo with no past history of malaria as controls ($n = 30$) (Wickramarachchi et al., 2006). Blood was drawn under aseptic conditions by venipuncture and serum samples were stored at −20 °C until further use.

Since most malaria infections are symptomatic in both endemic and non-endemic regions of Sri Lanka (Mendis

et al., 1990; Gunewardena et al., 1994) and malaria episodes are routinely confirmed by thick and thin blood smear microscopy prior to treatment, the self-reported number of previous malaria infections (including *P. vivax* and *P. falciparum*) was used to obtain the previous cumulative exposure to malaria of each patient. Age of the patient and duration between present and the penultimate malaria infection were also noted.

2.3. Recombinant p42 and p19 proteins

P. vivax C-terminal MSP-1 baculovirus recombinant proteins (Belem strain) (del Portillo et al., 1991) corresponding to PvMSP-1p42 (residues 1,325–1,704) and PvMSP-1p19 (residues 1,602–1,704) (Longacre et al., 1994; Holm et al., 1997) were used for ELISA.

2.4. Determination of total anti-p42 and anti-p19 antibodies by indirect ELISA

Serum antibodies specific for PvMSP-1p42 and PvMSP-1p19 were assayed separately by ELISA using recombinant proteins p42 and p19, respectively, as previously described (Wickramarachchi et al., 2006). Each test serum sample was assayed against the proteins in duplicate with normal human sera as control. Checkerboard titrations determined the optimal dilutions of reagents used in these ELISAs, i.e., 0.2 μg/ml antigen, serum samples at 1:100 dilution. The positive cut-off value was calculated as the mean OD value of the normal controls plus 2 SD. For each sample the mean OD value obtained at a dilution of 1:100 was considered to be a measure of the magnitude of both anti-p19 and anti-p42 antibody responses. Endpoint titers were determined using twofold serial dilutions from 1:100 to 1:51,000 for each serum sample.

2.5. Subclass ELISA

Isotype-specific mouse-anti human monoclonal antibodies (mAbs) were used in an ELISA for the quantification of IgM and IgG isotypes (IgG1, IgG2, IgG3 and IgG4) (Wickramarachchi et al., 2006). Responder sera for total p19 and p42 antibodies from each test area were randomly selected for isotype determination. Microtiter plates were coated with antigen at 4 μg/ml for IgM and 10 μg/ml for IgG isotypes. Isotype-specific mouse anti-human mAb (Sigma, USA; IgG1 mAb HP-6001, IgG2 mAb HP-6002, IgG3 mAb HP-6050, IgG4 mAb HP-6025 and IgM mAb MB-11) were used at dilutions of 1:2,000 (IgM) and 1:3,000 (IgGs). Positive cut-off values for each isotype were determined as described above.

To adjust the affinity differences between the IgG isotype-specific mAbs, the specific OD values were adjusted by calibrating the assay using a reference serum (human standard serum NOR-01; Nordic Immunology). 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 (Wickramarachchi et al., 2006).

2.6. Statistical analysis

Statistical analyses of data were performed using SPSS 11 for windows (SPSS Inc., USA) and Epi Info 6 (version 6.04b to c upgrade; CDC, USA and WHO, Switzerland) computer programs. Proportions of responders (total and isotype-specific) for independent samples were compared using the Chi-square test and paired comparisons were made with the McNemar test. Comparisons of antibody levels of independent samples were performed using *t*-test, ANOVA, Mann–Whitney *U* test and Wilcoxon Signed Rank test, as appropriate. Paired *t*-test and Kruskal–Wallis H test were used for dependant samples. Associations between antibody responses and prevalence with host factors were derived using the Spearman and Pearson correlation coefficients, and the Chi-square for linear trend, respectively. The significance level was set at $P < 0.05$.

3. Results

3.1. Total (IgM + IgG) antibody responses to p42 and p19

Total anti-p42 and anti-p19 antibody responses (IgM + IgG) in 298 and 323 sera, respectively, from the three test areas were analyzed using ELISA. Antibody prevalence (percentage of antibody-positive sera) for p42 and p19, respectively, was 71% and 57% from Anuradhapura, 73% and 59% from Kataragama and 77% and 63% from Colombo (Fig. 1A). The antibody prevalence to the two proteins at the individual level tend to respond more to p42 than to p19 (McNemar test, $P < 0.01$) in all test areas. This was also reflected at the population level in each test area (Chi-square test; $P < 0.05$). No significant differences were apparent in p42 and p19 antibody prevalence (Chi-square test; $P > 0.05$), total antibody levels (one-way ANOVA; $P > 0.05$) and end-point titers (Kruskal–Wallis H test; $P > 0.05$) either between the two endemic areas or between endemic and non-endemic areas (Table 1). Specific antibody levels and corresponding end point titers for each antigen were highly correlated in all test areas (Pearson's Correlation test, $r > 0.6$, $P < 0.000$). A highly significant correlation existed between end point titers to the two proteins in all test areas (Spearman's Correlation test, Anuradhapura: $r = 0.694$; Kataragama: $r = 0.311$; Colombo: $r = 0.623$; $P < 0.01$). However, p42 titers were significantly higher than those of p19 in the three test populations (Wilcoxon Signed Ranks test, $P < 0.001$).

The proportion of patients responding to either of the antigens were 72%, 74% and 83% from Anuradhapura, Kataragama and Colombo, respectively, whereas 53%, 58% and 69%, respectively, responded to both proteins (Fig. 1B). Further, 17% of individuals from Anuradhapura, 16% from Kataragama and 14% from Colombo preferentially recognized p42, while the reverse was true for only 2%, 1% and

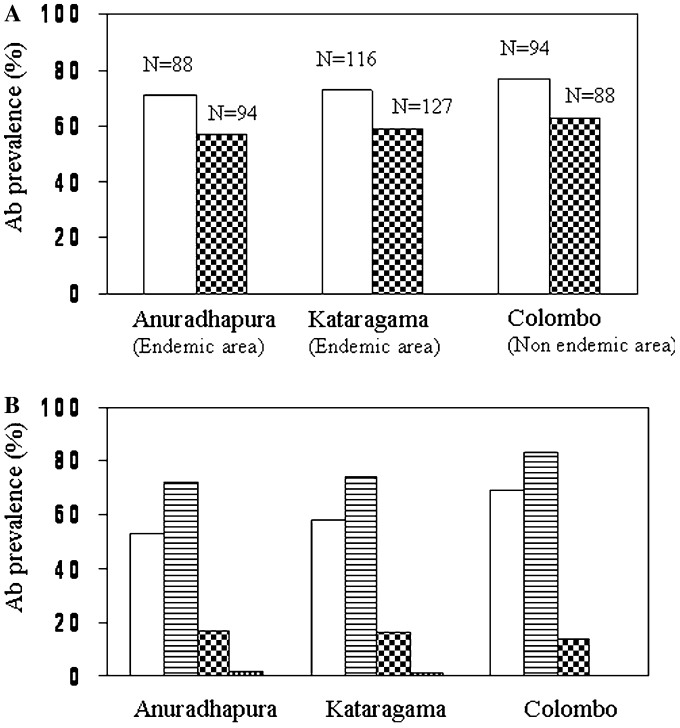

Fig. 1. (A) Anti-PvMSP-1p42 and p19 antibody prevalence of different study areas. Sample numbers in each group are indicated above the bars. p42 (□), p19 (▩). (B) Antibody prevalence to PvMSP-1p42 and p19 in each individual. Percentage of individuals that responded to both antigens (□), either one of the antigens (▤), only to p42 (▩) and only to p19 (▥).

0% from Anuradhapura, Kataragama and Colombo, respectively. None of these parameters differed significantly amongst the three study areas (Chi-square test; $P > 0.05$).

3.2. Isotype-specific antibody responses

Serum samples that were positive for total anti-p42 and anti-p19 immunoglobulins (IgM + IgG) were characterized for their IgM and IgG subclass responses to the two antigens.

IgM responses to p42 only were observed in 47%, 27% and 11% of individuals from Anuradhapura, Kataragama and Colombo, respectively, compared with anti-p19 IgM of 9%, 2% and 4%. Anti-p19 IgM prevalence at the individual level among endemic residents was significantly lower compared to p42 (McNemar test, $P < 0.01$), but not in the non-endemic population (McNemar test, $P > 0.05$).

Specific IgM responses to p42 revealed significant differences in prevalence between Anuradhapura and Colombo (Chi-squared test, $P < 0.05$), whereas anti-p19 IgM prevalence of residents from both endemic areas showed significantly lower values compared with their non-endemic counterparts (Chi-squared test, $P < 0.01$; Table 2). Nevertheless, no significant differences in the magnitude of IgM to either antigen amongst the three test sites was apparent (one-way ANOVA; $P > 0.05$; Table 2).

Among the four isotypes evaluated, generally elevated cytophilic IgG1, comparable IgG2 and IgG3, and discernible low IgG4 responses, were apparent in individuals of all test areas. The percentage of responders for IgG1 and IgG3 to p42 were 64% and 50% from Anuradhapura, 90% and 85% from Kataragama and 81% and 77% from Colombo, respectively. The corresponding percentages for p19 were 44% and 53% from Anuradhapura, 94% and 92% from Kataragama and 92% and 90% from Colombo, respectively. No disparities were detected in any test area between the prevalence of IgG1 and IgG3 antibodies to both antigens tested (Chi-square test, $P > 0.05$). Furthermore, in each individual the prevalence of IgG1 and IgG3 against each antigen within each test area did not significantly differ (McNemar test, $P > 0.05$). Nevertheless, most individuals showed a bias towards higher levels of anti-p19 IgG1 compared with IgG3 responses (Paired-samples *t*-test, $P < 0.01$), whereas a bias towards higher anti-p42 IgG1

Table 1
Prevalence, antibody level and reciprocal end point titers of total (IgM + IgG) anti-p42 and -p19 of acute *Plasmodium vivax* patients from the three study areas

Area	p42[a]			p19[a]		
	Prevalence %	Antibody level[b]	Reciprocal end-point titer[c]	Prevalence %	Antibody level[b]	Reciprocal end-point titer[c]
Endemic						
Anuradhapura	71	1.485 (±0.07)	6,400 (400, 51,200)	57	1.172 (±0.041)	6,400 (1,600, 5,600)
Kataragama	73	1.314 (±0.045)	6,400 (1,600, 51,200)	59	1.223 (±0.046)	3,200 (400, 25,600)
Non-endemic						
Colombo-total	77	1.443 (±0.06)	51,200 (6,400, 51,200)	63	1.276 (±0.059)	9,600 (1,600, 25,600)
PNE[d]	63	1.390 (±0.102)	25,600 (5,600, 51,200)	42	1.183 (±0.094)	6,400 (400, 19,200)
PE[e]	86	1.475 (±0.076)	51,200 (6,400, 51,200)	84	1.323 (±0.058)	12,800 (1,600, 25,600)

[a] The number of individuals in each test group was 88 and 94 from Anuradhapura, 116 and 127 from Kataragama and 94 and 102 from Colombo, for p42 and p19, respectively.

[b] The mean OD_{405nm} value obtained at a serum dilution of 1:100 was considered as a measure of the level of the anti-p19 and -p42 total antibody response of each individual. The cut-off value was the mean OD_{405nm} value plus 2 SD of the healthy individuals from Colombo, with no past history of malaria The antibody level of each test area is expressed as means ± SEM.

[c] The reciprocal end point titer of each test area is expressed as the median with lower and upper quartiles in parentheses.

[d] Previously not exposed to malaria.

[e] Previously exposed to malaria.

Table 2
Prevalence and levels of anti-p42 and -p19 IgM antibody responses of acute *Plasmodium vivax* patients from the three study areas

Area	P42			p19		
	N	Prevalence (%)	IgM level[a]	*N*	Prevalence (%)	IgM level[a]
Anuradhapura	45	60[b]	0.792 (±0.068)	55	29	0.468 (±0.036)
Kataragama	46	74[b]	0.781 (±0.06)	48	50	0.502 (±0.041)
Colombo	44	84	0.810 (±0.053)	55	74	0.502 (±0.026)

[a] The mean OD_{405nm} value obtained at a serum dilution of 1:10 was considered as a measure of the antibody level of the anti-p19 and -p42 IgM isotype response of each individual. The cut-off value was the mean OD_{405nm} value plus 2 SD for the healthy individuals from Colombo with no past history of malaria. The IgM level of each test area is expressed as means ± SEM in parentheses.
[b] The IgM prevalence among endemic residents against p42 was significantly higher compared with p19 (McNemar test, $P < 0.01$).

responses compared with IgG3 existed only for Colombo residents (Paired-samples *t*-test, $P < 0.01$).

3.3. Associations between total antibody responses and host factors

There was no correlation between total p42 or p19 antibody magnitude or end point titers in any of the test areas, either with age of the patient, their parasite density or the duration between current and the penultimate *P. vivax* infections (Pearson and Spearman's Correlation coefficient, $P > 0.05$).

Patients were classified into four groups based on past exposure to malaria as follows: PNE (experiencing their first clinical malaria infection), 1–2 infections, 3–5 infections and >5 previous infections. PNE patients from Colombo (p42: $N = 41$; p19: $N = 50$) showed significantly lower antibody prevalence (p42: 63%; p19: 42%) compared with PE patients from Colombo (p42: 86%; p19: 84%) (p42: Chi-square 6.32, $P = 0.012$; p19: Chi-square 19.79, $P = 0.000$). Twenty-one percent of PNE patients responded preferentially to p42 (McNemar test, $P = 0.016$), whereas none recognized only p19. The corresponding values for PE patients from Colombo were 8% and 0%. The percentage of PNE patients who responded to both or either of the antigens were 47% and 68%, respectively, and for the PE group 89% and 97%, respectively. Nevertheless, both PNE and PE patients from Colombo exhibited comparable total antibody magnitudes (*t* test, $P > 0.05$) and endpoint titers (Mann–Whitney *U* test, $P > 0.05$) for both antigens tested (Table 1). Nevertheless, there were significantly more individuals with antibodies to both p42 and p19 in the two endemic areas and in PE compared with PNE Colombo residents (Chi-square test, $P < 0.05$).

3.4. IgM, IgG1 and IgG3 isotype responses to p42 and p19: association with previous exposure to malaria

No correlations were observed with age, parasite density or previous exposure for p42- and p19-specific IgM, IgG1 and IgG3 isotype levels in any of the three areas tested (Pearson and Spearman's Correlation coefficient, $P > 0.05$).

Individuals from Kataragama showed a negative trend between anti-p42 IgM prevalence and increasing exposure (Chi-square for linear trend, 4.04, $P = 0.04$). Similarly, negative trends existed between anti-p19 IgM prevalence and increasing exposure among individuals from both endemic areas only (Anuradhapura: Chi-square for linear trend, 4.2, $P = 0.04$; Kataragama: Chi-square for linear trend, 8.38, $P = 0.004$). Nevertheless, PE individuals from Colombo showed a considerably lower prevalence of anti-p19 IgM (64%, $N = 33$) compared with PNE individuals (90%, $N = 21$), although this disparity was not statistically significant (Chi-squared test, 3.52, $P = 0.06$). There was no association between the number of anti-p42 or anti-p19 IgG1 and IgG3 isotype responders and previous exposure in any test area.

For the analysis of p42- and p19-specific IgM, IgG1 and IgG3 variation associated with previous exposure to malaria, individuals were classified into two groups in each test area: (i) those having IgM responses, with or without IgG (IgM [±IgG]) and (ii) those showing only IgG responses (Fig. 2).

IgM-restricted responses comprised a low percentage of individuals in both endemic and non-endemic populations for both antigens. However, the proportion of IgG-restricted compared with IgM (±IgG) p19 responders in endemic areas (54% and 51% for Anuradhapura and Kataragama, respectively) differed significantly from non-endemic Colombo residents (21%) (Chi-squared test, 10.48, $P < 0.01$; Fig. 2). Interestingly, there were no such differences between p42 IgG-restricted and IgM (±IgG) responses with regard to endemicity (Anuradhapura: 16%, Kataragama: 20% and Colombo: 15%) (Chi-square test, $P > 0.05$; Fig. 2). While the percentage of individuals with combined p19-specific IgM and IgG antibodies was highest in PNE individuals from Anuradhapura (71%; Fig. 2), this parameter was markedly reduced following exposure to malaria. In contrast, the percentage of Anuradhapura residents with IgG-restricted responses was maximal in individuals with more than five past malaria infections (100%), (Chi-square for linear trend; 5.87, $P = 0.015$). A similar pattern of prevalence between p19-specific combined IgM (±IgG) and IgG-restricted groups was apparent in residents of Kataragama (Chi-square for linear trend; 9.82, $P = 0.002$). Nevertheless, the trend towards p19 IgG-dominated immune responses with increasing exposure to malaria was not observed in non-endemic residents, who showed a high prevalence of IgM (±IgG) antibody

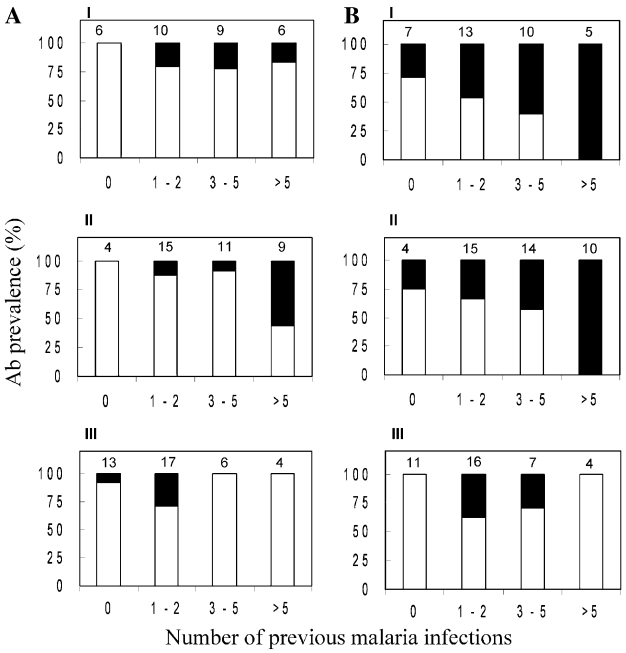

Fig. 2. Pattern of isotype-specific antibody prevalence to PvMSP-1p42 (A) and PvMSP-1p19 (B) against different numbers of previous malaria infections in Anuradhapura (I), Kataragama (II) and Colombo (III). Individual responses were categorized as IgM responses with or without IgG (□), or IgG restricted responses (■). The number of individuals tested in each past exposure group is indicated above the bars.

responses (62–100%), irrespective of previous exposure to malaria (Chi-square for linear trend; 0.23, $P = 0.629$). A similar pattern of subclass-specific shift towards cytophilic IgG antibodies dominated the p42 immune response with increasing exposure to malaria in Kataragama residents only (Chi-square for linear trend; 5.89, $P = 0.015$) (Fig. 2), although this phenomenon was less marked than the p19-specific switch to cytophilic IgG. The prevalence of the IgG-restricted response, though statistically not significant, was higher in the Kataragama group with more than five past infections than the corresponding group in Anuradhapura, reflecting the disparity in exposure of these two group (mean exposure: 8 ± 0.8 in Kataragama, 3 ± 0.3 in Anuradhapura).

3.5. Comparison of antibody responses to *P. vivax* asexual stage vaccine candidate antigens (p42, p19 and AMA-1)

To determine whether the shift of isotype repertoire towards functionally important IgG isotypes is an intrinsic property of p19, natural antibody responses against *P. vivax* Apical membrane Antigen-1 (Wickramarachchi et al., 2006), using the same battery of sera, was also compared with these data. This analysis explored the nature of the naturally acquired antibody response of each *P. vivax*-infected patient with respect to three different asexual stage vaccine candidates (i.e., PvMSP-1_{42}, PvMSP-1_{19} and PvAMA-1) during the same episode of malaria. Both C-terminal MSP-1 and AMA-1 antigens showed strong correlations with respect to their IgM-, IgG1- and IgG3-specific antibody responses (Fig. 3). Each individual was analyzed to elucidate whether he/she showed the same isotype profile to the three different antigens tested. For this analysis, individuals from both endemic test areas were pooled into one group ($N = 38$). The number of individuals with a restricted IgM response was negligible compared with the other two types in both endemic and non-endemic areas. 15% and 24% of individuals from pooled malaria endemic areas showed IgG-restricted antibody responses, and a combined isotypic response (i.e., IgM + IgG response), respectively, against all antigens tested. The IgG-restricted antibody responses of the rest of the individuals (61%) were limited to either a single or combination of two antigens. The corresponding percentages of individuals from non-endemic Colombo ($N = 21$) were 0%, 71% and 29%, respectively, showing significant differences compared with endemic residents (Chi-square test, $P < 0.05$). An IgG-restricted response was absent in those individuals suffering their first malaria infection in the endemic population (Fig. 4). However, 43% of individuals with more than five past malaria infections from this endemic population had IgG-restricted responses to all three antigens tested (Fig. 4). The IgG-restricted antibody response against either p19 or AMA-1 manifested in 86% of individuals from the latter category. Hence, with increasing exposure to malaria in the endemic areas a shift in their antibody response from a combination of non-cytophilic and cytophilic isotypes [IgM + (IgG1 + IgG3)] towards cytophilic isotypes (IgG1 + IgG3) was evident significantly for p19 and AMA-1 compared with those of p42.

4. Discussion

This study reports for the first time a cross-sectional direct comparison of naturally acquired antibody responses to MSP-1p42 and MSP-1p19, the C-terminal processing products of *P. vivax* Merozoite Surface Protein-1, two of the current leading vaccine candidates against vivax malaria. This data also represents evidence of natural immune responses using recombinant proteins produced in the baculovirus/insect cell expression system (Longacre et al., 1994; Chitarra et al., 1999; Pizarro et al., 2003). Importantly, compared with PfMSP-1 C-terminal antigens, similar immuno-epidemiological data regarding antibody responses to PvMSP-1 is scarce (Soares et al., 1997, 1999). Implications of this study are widely applicable to C-terminal PvMSP-1-based vaccine development in most vivax-endemic regions, where transmission rates are defined as low (Mendis et al., 2001).

The higher prevalence of p42 antibodies and higher numbers of individuals responding exclusively to p42 and not to p19 among individuals not previously exposed, indicate that this antigen is clearly more immunogenic in malaria-naïve or less exposed individuals than those of p19 in natural infections. These findings confirm previous

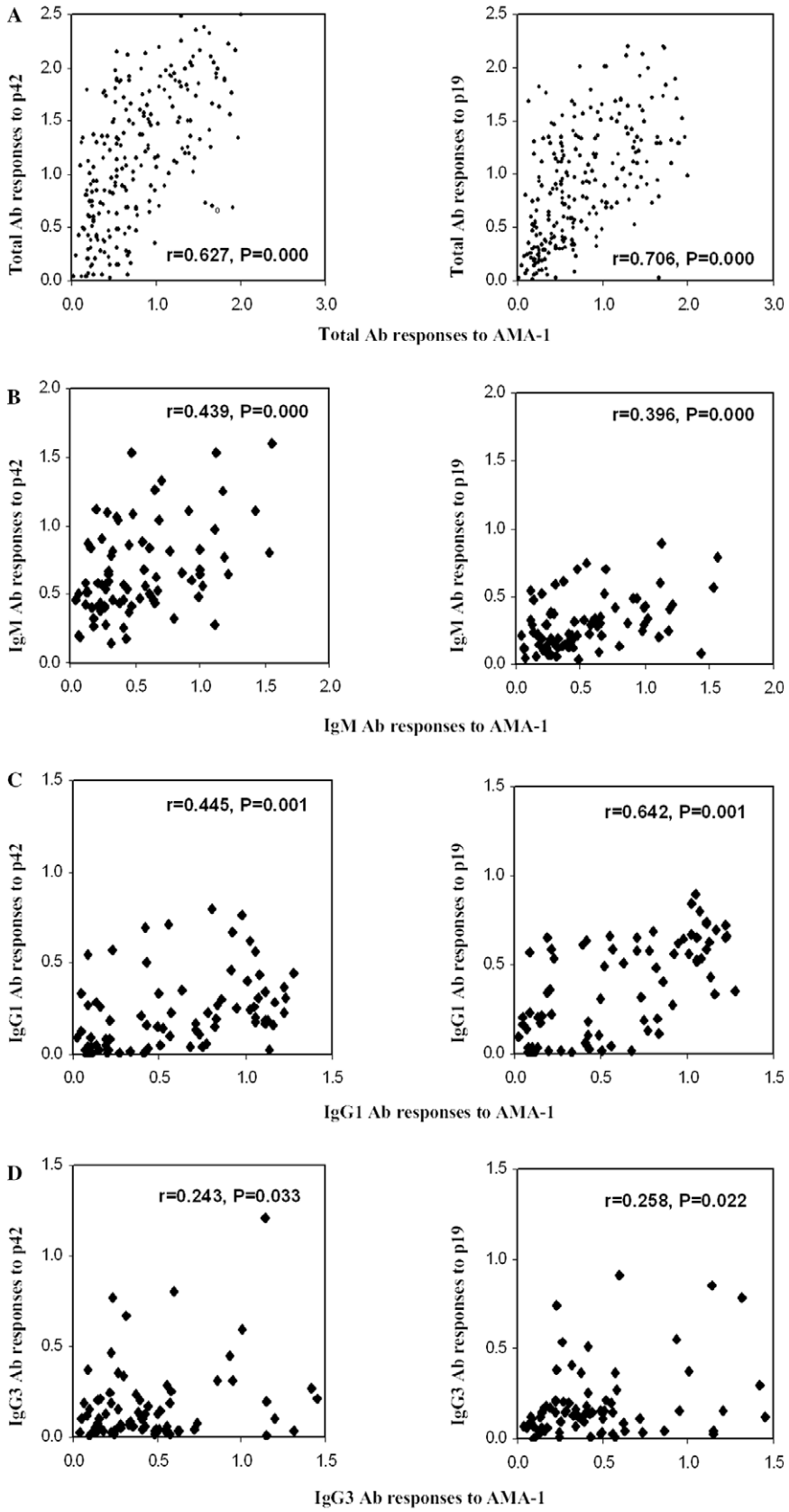

Fig. 3. Correlation between total (A) and isotype-specific (B–D) antibody responses to PvAMA-1, with those of PvMSP-1p42 and PvMSP-1p19, separately. Respective Pearson's correlation coefficients and *P* values are indicated in each graph.

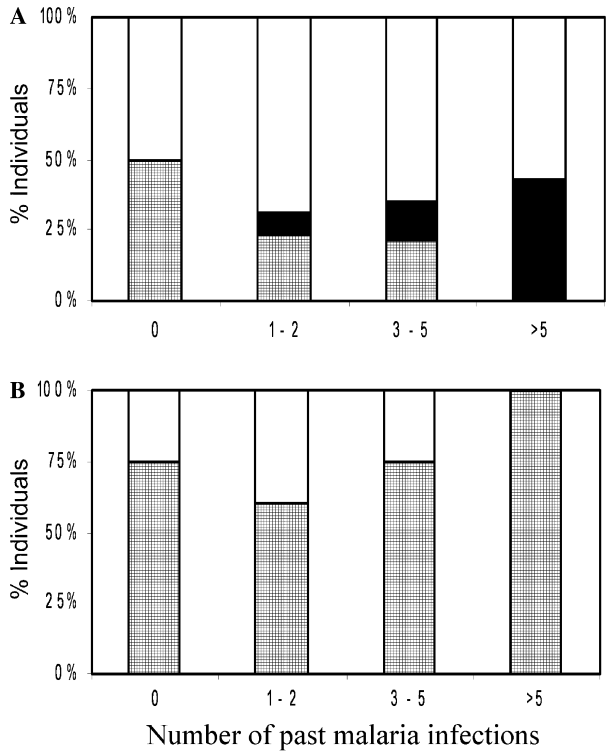

Fig. 4. Distribution of IgG restricted antibody responses against the three *P. vivax* vaccine candidates, MSP-1p42, MSP-1p19 and AMA-1, of each individual in relation to previous exposure to malaria. Individuals from malaria endemic (A) and from non-endemic (B) areas. Four groups were established based on previous exposure to malaria of each individual: no previous infection, 1–2 previous infections, 3–5 previous infections and >5 previous infections. In both (A) and (B), individuals with IgG restricted response to all three proteins, (▤) individuals with a combination of IgM + IgG response to all three proteins, and (□) individuals with IgG restricted response limited to at least one of the three proteins.

reports on the C-terminal region of PvMSP1, indicating that the PvMSP-1p42 harbors a hypervariable immunogenic block flanked by conserved regions of 98–100% homology (del Portillo et al., 1991; Gibson et al., 1992; Manamperi, A., 2002. Ph.D. thesis. University of Colombo, Sri Lanka; Putaporntip et al., 2002). Since the MSP-1p19 domain is cysteine rich, with multiple disulphide bonds forming two EGF-like domains, where B-cell epitopes appear to be almost exclusively conformational (Soares et al., 1997), it has been argued that the complex MSP-1p19 3D structure may be poorly processed for T-cell epitopes (Egan et al., 1997). However, since the baculovirus p19 recombinant antigen from *P. cynomolgi* MSP1 is highly immunogenic when purified and used to vaccinate primates (Perera et al., 1998), this relatively small C-terminal peptide derived from a large 200 kDa precursor may not be well exposed to the immune system in its natural context on the merozoite surface. In contrast, the secondary structure predicted for the hypervariable block of PvMSP-1p42 suggests this domain may form a highly exposed immunogenic loop and it comprises many cross-reactive B-cell epitopes among different haplotypes (Manamperi, A., 2002. Ph.D. thesis. University of Colombo, Sri Lanka).

Anuradhapura and Kataragama are considered typical *P. vivax* endemic areas in Sri Lanka (Gunewardena et al., 1994). During the study period the annual parasite incidence was 5–40 and 80–160 per 1,000 population in Anuradhapura and Kataragama, respectively (Briët et al., 2003). Residents of Colombo, where there is no malaria transmission, are infected while visiting endemic areas and those exposed to multiple malaria infections, frequent endemic areas due to their occupation (Fonseka and Mendis, 1987). Thus, comparable p42- and p19-specific antibody prevalence in malaria endemic and non-endemic areas was unexpected; as our working hypothesis was that this parameter would be lower in Colombo residents.

Manamperi (Manamperi, A., 2002. Ph.D. thesis. University of Colombo, Sri Lanka) reported that the PvMSP-1p42 hypervariable region of 38 amino acids appeared to be as variable in Kataragama within 3 months, and immune responses against this region were correlated with the current infection allele, regardless of previous infection history. This response seems to be characterized by multiple cross-reactive epitopes and low-affinity antibodies, suggestive of an immature response, and may account for the findings reported here, showing a considerably higher percentage of sera from endemic areas that preferentially contain anti-p42 IgM compared with anti-p19 IgM. These results suggest that the hypervariable region may have a tendency to evoke a preferential immature IgM response to each new hypervariable region allele in sequential infections, while the response to the highly conserved anti-p19 (Carter and Mendis, 2002) matures more rapidly into an IgG response. IgM-dominated response to both PvMSP-1p42 and PvMSP-1p19 in the PE individuals from Colombo may explain the lack of immune memory due to the long time lapse between the present and the penultimate malaria infections compared with their endemic counterparts (Ranawaka et al., 1988; Carter and Mendis, 2002).

The IgG isotype profiles described here were similar to those of *P. vivax*-endemic areas in Brazil and appeared to be intermediate between high transmission *P. falciparum*-endemic areas in Africa (Shi et al., 1996; Sarthou et al., 1997) and non-immune subjects from suburban South America (Ferreira et al., 1998).

Although there was no serological data for children in this study (due to ethical and pragmatic reasons), the very low inoculation rates (Mendis et al., 1990) and heterogeneous exposure among different age groups in Sri Lanka (Mendis et al., 1992) make it unlikely that such an age correlation exists in these settings. Low transmission rates in Sri Lanka (Mendis et al., 1990) can also explain the lack of association between antibody levels specific for p42 and p19, and reduction of parasite burdens and clinical episodes, as seen for the corresponding *P. falciparum* antigens (Al-Yaman et al., 1996; Shi et al., 1996).

The marked switch of anti-p19 IgM to functionally important cytophilic IgG1 and IgG3 antibodies with increasing exposure in both endemic areas, is consistent with the notion that cytophilic antibodies play a prime role in protective mechanisms involving cooperation with monocytes (Bouharoun-Tayoun and Druilhe, 1992; Bouharoun-Tayoun et al., 1995). In contrast to p19, p42 antibodies principally reflected a primary IgM response irrespective of the patient's previous exposure. While a significant p42 isotype switch to IgG was restricted to Kataragama indicative of the necessity of sustained exposure, the data represents a maximal estimate, since this antigen also includes the p19 domain, making it difficult to gauge the p42-specific contribution. These data suggest that a strain-specific PvMSP-1p42 "immature" antibody response, with a high proportion of IgM antibodies directed against the p42 polymorphic region, may interfere with a p19 "protective" isotype shift. Overall, this suggests that immunological "cross-talk" between epitopes of PvMSP-1p42 and p19 may interfere with the development of a mature IgG dominated protective antibody response, which may complicate vaccine development.

The analysis carried out with subclass-specific antibody responses against p42, p19 and AMA-1 of *P. vivax* of each individual from malaria endemic areas, clearly demonstrated the higher prevalence of IgG1 against more conserved antigens, AMA-1 (unpublished data) and MSP-1p19, compared with polymorphic MSP-1p42 (Manamperi, A., 2002. Ph.D. thesis. University of Colombo, Sri Lanka). The subclass prevalence pattern at both population and individual levels signify that the constituents of the serologic response against p19 and AMA-1 were distinctly different from that to p42, even in individuals with similar previous exposure to malaria. This subclass profile characterised for PvAMA-1 was similar to that previously reported from Brazil (Rodrigues et al., 2005). This indicates that the prevailing B-cell epitope repertoire to MSP-1p19 and AMA-1 was limited and that it is possible to encounter a majority of the B-cell repertoire during previous malaria exposure of endemic individuals. Further, although the local transmission intensity is comparably low, the frequency of infections of individuals residing in endemic areas was sufficient to maintain the immune memory to conserved antigens tested. It would be of interest to examine in more detail the subclass maturation of antibodies specific to different regions of these antigens as a function of transmission intensity.

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