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HUMAN IgG SUB-CLASS ANTIBODIES TO THE 19 KILODALTON CARBOXY TERMINAL FRAGMENT OF *PLASMODIUM FALCIPARUM* MEROZOITE SURFACE PROTEIN 1 ($MSP1_{19}$) AND PREDOMINANCE OF THE MAD20 ALLELIC TYPE OF MSP1 IN UGANDA.

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ABSTRACT

Objective: **To determine the natural human humoral immune responses to the 19 kilodalton carboxy terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 ($MSP1_{19}$), a malaria candidate vaccine antigen and to determine the prevalence of MAD20 and K1 alleles of *P. falciparum* MSP1.**

Design: **Community based cross-sectional study.**

Setting: **Atopi Parish, Apac District, Uganda, 1995.**

Subjects: **Three hundred and seventy four Ugandans between <1 and 70 years old provided serum samples.**

Main outcome measures: **IgG subclass antibodies by ELISA; MAD20 and K1 allelic types of MSP1 by PCR.**

Results: **Both the prevalence and the mean concentration of serum IgG1 , and to a lesser extent IgG3, antibodies increased with age. IgG2 or IgG4 antibodies were virtually non-existent. The cross-reactivity between the 4 sequence variants (E-KNG, E-TSR, Q-KNG and Q-TSR) of $MSP1_{19}$ was confirmed; however, a minority of sera preferentially recognised the KNG but not the TSR variants. All 33 *P. falciparum* isolates from different parts ofm Uganda carried the E-TSR (Mad20) allelic type and 3 isolates were mixed infections with E-TSR (MAD20) and Q-KNG (K1) allelic types, confirming the rarity of the K1 allele in Uganda.**

Conclusion: **There is a robust IgG1 antibody response to the malaria vaccine candidate antigen $MSP1_{19}$ which begins at an early age. Future cohort studies are necessary to estblish the impact of these antibodies on clinical immunity to malaria. The MAD20 allelic type of MSP1 id predominant in Ugandan *P. falciparum* isolates.**

INTRODUCTION

The merozoite surface protein (MSP1), a 195 kilodalton (kD) protein on the surface of merozoites which is processed into many shorter polypeptides prior to the invasion of the erythrocyte, is the subject of intense investigations aimed at developing *Plasmodium falciparum* blood-stage vaccines(1). Evidence from in vitro and challenge studies in mice and primates suggests that a vaccine based on MSP1 might have an immunoprophylactic effect against malaria(2-5). Immunisation with recombinant *Plasmodium yoelii* MSP1(6) or a DNA vaccine encoding *Plasmodium yoelii* MSP1(7) has protected mice against *P. yoelii* sporozoite challenge; immunised mice exhibited boosting of antibody responses after infection(8). MSP1 is composed of conserved, polymorphic and dimorphic sequences. Although the carboxy terminal 42 kD fragment is dimorphic, with the two alleles being classified as MAD20-like or K1-like(9), the 19 kD fragment is essentially conserved with only 4 amino acid differences between the two dimorphic types(10). This divergence has resulted in the existence of four possible sequence variants of $MSP1_{19}$ which have been successfully expressed in the yeast *Saccharomyces cerevisiae* as secreted polypeptides(11).

Antibodies to the carboxy terminus of MSP1 are believed to be responsible for much of the protective effect of antibodies to native MSP1 . Immunisation of rabbits with the carboxy terminal 42 kD portion of *P. falciparum* MSP1 induced antibodies, which inhibited parasite growth *in vitro*(12). Studies of West African children have demonstrated that the prevalence of antibodies to the C terminus of MSP1 was associated with resistance to clinical malaria and high parasitemia in young children(13,14). However, the correlation between responses to $MSP1_{19}$ and clinical immunity observed in West African children still remains to be confirmed in other field sites in Africa which have different malaria transmission patterns. To this end, we have begun to investigate immunological responses to $MSP1_{19}$ in Atopi Parish in northern Uganda.

In this paper, we describe IgG subclass responses to MSPl$_{19}$ in Atopi residents. The majority of these subjects possessed cross-reactive IgG1 antibodies against all four sequence variants of MSP1$_{19}$. We also observed that MAD20 was the predominant allelic type of MSPl present in Ugandan *P. falciparum* isolates.

MATERIALS AND METHODS

Study population and sample collection: Atopi Parish is located about 5 km west of Apac Town, 300 km north of Kampala. The population is predominantly of the Lango tribe and the major occupation is peasant agriculture. The description of the study population and preliminary malariometric data has been reported elsewhere (Egwang *et al*). During a cross-sectional survey carried out in December 1995, 1246 subjects were interviewed using a standard questionnaire and subjected to medical examination by a clinician. Blood samples from all subjects were collected in vacutainers containing EDTA. Thin and thick blood films were made for each sample, stained with Giemsa, and examined for malaria parasites under high power microscopy. Serum samples separated from blood were collected into fresh serum vials and transported on ice to Kampala where they were stored at -20 °C. Finger prick blood samples from children were collected onto filter papers (Whatman paper # l); the filters were air dried, stored individually in plastic envelopes, and kept at 4°C until genomic DNA was extracted. The demographic and parasitologic data for 374 subjects whose serum and parasite isolates were used for the immunological and parasite genotyping studies are presented in Table 1.

Antigens, immunochemicals and primers: Four variants of MSP1$_{19}$, the carboxy terminal fragment of *Plasmodium falciparum* merozoite surface protein, namely Q-TSR, Q-KNG, E-TSR, and E-KNG produced in recombinant *Saccharomyces cerevisiae* were kindly provided by Dr David Kaslow (Molecular Vaccine Section, National Institute of Health, Bethesda, MD, USA). Alkaline phosphatase conjugated murine monoclonal antibodies to IgGl, IgG2, IgG3, and IgG4 antibodies were purchased from Zymed Laboratories, Inc, South San Francisco, CA, USA and from The Binding Site Limited, Birmingham, UK. Primers and protocols for genotyping *P. falciparum* isolates at the MSP1 locus by the polymerase chain reaction (PCR) were provided by Dr. Lisa Ranford Cartwright (Institute of Cell, Animal, and Population Biology, University of Edinburgh, UK).

Enzyme-linked immunoadsorbent assays (ELISA): The E-KNG variant derived from the Palo Alto strain was chosen for initial studies and IgG subclass antibodies reacting with E-KNG were detected by ELISA. Immunolon 4 microtitre plates (Dynatech, UK) were coated overnight at 4°C with 1 µg/ml antigen in carbonate buffer. Plates were washed three times with phosphate buffered saline (PBS) containing 0.5 % v/v Tween 20 (PBS/T), blocked for one hour with 1% powdered skimmed milk in PBS/T (blocking buffer) and serum samples diluted 1:50 in blocking buffer were added to the plates in duplicate and incubated for one hour at 37°C. The plates were then washed three times in PBS/T and incubated for one hour at 37°C with alkaline phosphate conjugated mouse monoclonal antibodies against human IgG1, IgG2, IgG3, or IgG4 appropriately diluted in PBS/T. After the final wash, the reactions were developed by adding p-nitrophenyl phosphate (Sigma) and read at 405 nm after 30 minutes. Serum samples from 30 Europeans not previously exposed to malaria were employed as negative controls to obtain the cut-off value for positive antibody reactions. The cut-off value was calculated as the mean optical density (OD) plus two standard deviations of the European sera.

Competition ELISA: We identified two groups of serum samples: those which reacted strongly with all the variants, and those which reacted strongly with only E-KNG and Q-KNG variants when IgGl antibodies were measured. Competition ELISA was therefore carried out in order to confirm the cross-reactivity of IgGl antibodies against MSPl$_{19}$ sequence variants in the majority of serum samples. Plates were pre-coated with Q-KNG or E-KNG and blocked as described above. Selected serum samples in both categories were pre-incubated at room temperature with increasing quantities of Q-TSR in carbonate buffer for 5 hours and then added to the wells of the pre-coated microtitre plates. The plates were washed three times in PBS/T followed by incubation for one hour at 37°C with 100 µl of alkaline phosphate conjugated mouse monoclonal antibodies against human IgGl. All subsequent steps including development with the substrate were as described above for ordinary ELISA.

Parasite genotyping for MSP1 Block 17: P. falciparum DNA was extracted from filter papers, slides, or red blood cell pellets as described(15-17). We obtained *P. falciparum* isolates from other regions of Uganda either as slides (Kabarole) or as red blood cell pellets (Kampala). PCR primers used for amplifying

Table 1

Demographic and parasitological data for the subjects who provided sera for immunological studies of MSP1$_{19}$

Age (years)	N	Sex		*P. falciparum*	
		Male	Female	Prevalence (%)	Density (GM parasites/µl blood)
<1	4	3	1	100.0	60.0
1-5	39	18	21	92.3	1272.7
6-8	61	35	26	80.3	899.1
9-10	41	18	23	80.5	300.3
11-14	49	32	17	63.8	296.8
15-20	49	19	30	39.6	314.4
21-30	36	16	20	52.8	125.8
31-40	40	17	23	45.0	151.4
>40	53	29	24	51.9	137.8
Total	372	187	185	64.1	327.9

MSPl block 17 included primers l G (5'-AAS/GAT/ATY/TTA/AAT/TCA/CG- 3') and lH (5'-TT/ATT/TTC/GTT/ACA/AGT/AGG-3') for the first outer dimorphic PCR. Nested PCR using the outer PCR product as template was carried out using MAD20- and K1-specific forward primers designated 1A and 1B, respectively, as well as a common reverse primer 1C. The sequences of 1A, 1B, and 1C were 5'-GAG/AAG/TTC/CCA/TCA/TCA/CCA/C-3', 5'-GAA/TTG/CTG/ATT/TAT/CAA/CAG-3', and 5'-CAT/CTA/AAT/GTC/TGAIAAC/ATC/C-3', respectively. Outer PCR was carried out using 1 µl of the *P. falciparum* DNA template in a 50-µl reaction volume containing 1.5 mM $MgCl_2$, 200 µM dNTP mix, 1.0 µg/µ1 primers, and 2.5 units of Taq polymerase (Promega) using the following conditions: pre-PCR incubation at 95°C for 5 min followed by incubation at 50°C for 5 min, followed by 39 cycles of 95°C for 1 min, 50°C for 45 sec, and 72°C for 1 min; during the last cycle, the extension at 72°C was for l0 minutes. Nested PCR was carried out using the inner primers 1A, 1B, and 1C at 1.0 µg /µl and outer PCR product as template. In this case, pre-PCR involved incubation of the samples at 94°C for 5 min followed by incubation at 54°C for 5 min; PCR consisted of 40 cycles, each cycle in turn consisting of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min; during the last cycle the extension at 72 °C was again for 10 minutes. Ten µl of PCR products were run on a 2.5% agarose gel, stained with ethidium bromide, and photographed under UV transillumination.

Data analysis: Positive sera were those whose ODs were greater than the normal range (mean ± 2SD) for control European sera. Mean OD values were compared using the two sample Student's t test for equal variances. Proportions were compared by obtaining pooled proportions for unpaired samples. Possible associations between antibody response and age were tested using the chi-square test for trend analysis.

RESULTS

Increasing prevalence and concentration of antibodies to $MSP1_{19}$ with age: Three hundred and seventy four serum samples were tested in ELISA for IgG subclass reactivity with the E-KNG sequence variant of $MSPl_{19}$. The prevalence and the mean concentration of IgG1 and IgG3 antibodies generally increased with increasing age (Figure 1, panels a and c; Table 2). Robust IgG1 responses seen in children 0-1 years old could be attributable to maternal antibodies; data from these children were excluded from the analyses. The prevalence of IgG1 antibodies in children 1-5 years old was lower than in subjects >14 years old (13.9 versus 61.4 %). This difference was statistically significant ($p < 0.001$). Mean OD values for IgG1 increased with age and, in general, individuals older than 14 years old had significantly elevated IgG1 levels. IgG3 antibodies, by contrast, had a delayed evolution, with robust responses beginning to appear only in children above seven to eight years old (Figure 1). Mean IgG3 levels remained low throughout the age groups and were significantly elevated only in individuals >40 years old (Table 2). Very few sera contained IgG2 or IgG4 antibodies to $MSP1_{19}$ and this did not change with age.

Figure 1

IgG subclass responses to $MSP1_{19}$ (E-KNG) as a function of age in a study population from Atopi Parish, Apac District in Northern Uganda. Panel (a), IgG1; panel (b), IgG2; panel (c), IgG3; and panel (d), IgG4. The age groups were defined as 1= 0-1 years, 2 = 1-2 years, 3 = 2-3 years, 4 =3-4 years, 5 =4-5 years, 6 = 5-6 years, 7 =6-7 years, 8 = 7-8 years, 9 = 8-9 years, 10 =10 years, 11=10-12 years, 12 = 12-14 years, 13 =14-16 years, 14 =16-20 years, 15 = 20-30 years, 16 =30-40 years, 17 =>40 years.

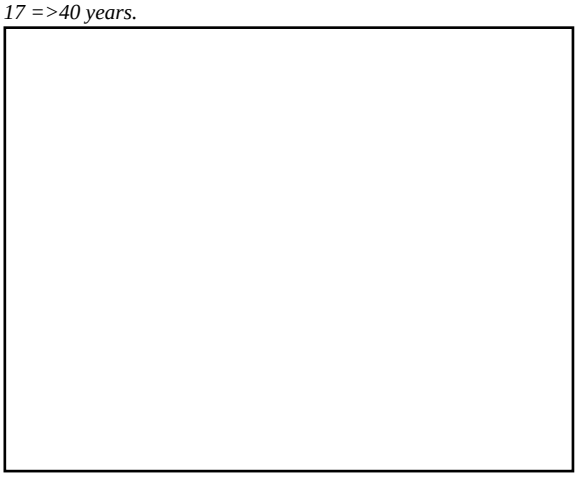


Table 2

IgG1 and IgG3 subclass antibodies to $MSP1_{19}$ in sera of Atopi parish residents.[a]

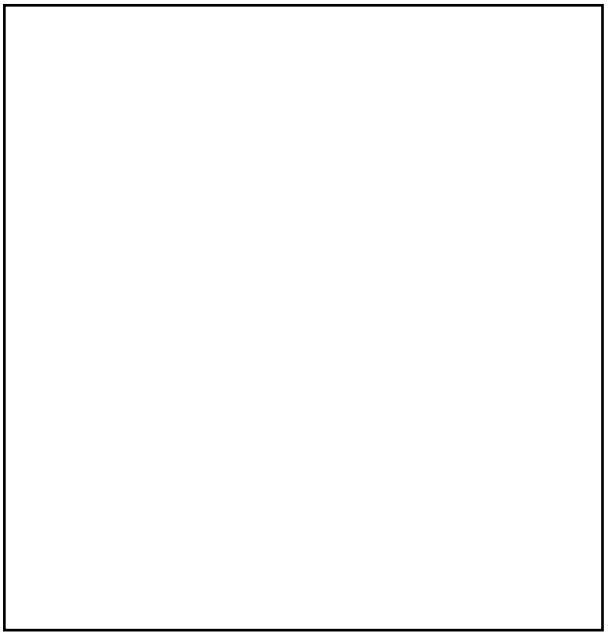
Age	IgG1			IgG3		
(years)	N	Mean OD (SD)	Prevalence (%)	N	Mean OD (SD)	Prevalence (%)
<1	4	1.107 (1.287)	50.0	5	0.308 (0.239)	40.0
1-5	36	0.352 (0.625)	13.9	39	0.183 (0.097)	38.5
6-8	54	0.375 (0.569)	22.2	55	0.230 (0.198)	45.5
9-10	29	0.439 (0.645)	33.3	36	0.304 (0.428)	55.6
11-14	42	0.612 (0.888)	31.1	41	0.361 (0.538)	53.7
15-20	44	1.024 (0.991)[b]	61.4[b]	41	0.344 (0.299)	75.6[b]
21-30	22	0.380 (0.464)	31.8	25	0.374 (0.284)	68.0[b]
31-40	22	0.519 (0.711)	31.8	22	0.383 (0.505)	59.1
>40	22	1.511 (1.287)[b]	63.6[b]	20	0.719 (0.834)[b]	85.0[b]

[a]Values are means with standard deviations SD in parenthesis and prevalence estimates of serum samples positive for antibodies.
[b]Significantly greater ($P < 0.05$) from the value for children in the age group 1-5 years.

Antibodies to MSP1$_{19}$ E-KNG cross-react with Q-TSR: In order to ascertain whether all the serum samples reacted equally strongly with other MSP1$_{19}$ variants; thirty six sera from adults with robust IgG1 responses against E-KNG were tested against all the sequence variants (E-KNG, E-TSR, Q-KNG and Q-TSR). None of these sera were able to discriminate between proteins carrying Q or E, i.e. OD values were closely correlated for Q-TSR versus E-TSR and values for Q-KNG were closely correlated with those for E-KNG (Figure 2a, 2b). However, three sera preferentially bound to KNG rather than TSR (Figure 2c, 2d). In several experiments, the three sera reacted with an OD < 0. 5 against TSR variants while reacting with an OD ~ 2.0 against KNG variants.

Figure 2

OD values for IgG1 reactivity against different MSP1$_{19}$ sequence variants are correlated. Panel (a), Q-TSR versus E-TSR; panel (b), Q-KNG versus E-KNG; panel (c), Q-KNG versus Q-TSR; and panel (d), E-TSR versus E-KNG. Note the discordance between recognition of Q-KNG and Q-TSR in panel (c) for one serum sample (arrow head) and between recognition of E-KNG and E-TSR in panel (d) for two serum samples (arrow heads).



Cross reactivity of IgG1 antibodies to TSR and KNG was confirmed by competition ELISA (Figure 3). Sera were pre-incubated with soluble Q-TSR and then added to plates pre-coated with Q-KNG. Q-TSR inhibited binding to Q-KNG for two serum samples (795 and 606). These two sera, representative of the majority of serum samples, bound equally to Q-KNG and Q-TSR. By contrast, the binding of two other serum samples (997 and 395) to Q-KNG was unaffected by pre-incubation with Q-TSR; these sera reacted with Q-KNG but not to Q-TSR confirming the lack of cross-reactivity of IgGl anti-Q-KNG with the Q-TSR variant.

Figure 3

Competition ELISA for IgG1 reactivity against Q-KNG after pre-incubation with various amounts of Q-TSR. There was competitive inhibition between Q-KNG and Q-TSR for samples 795 and 606, and lack of competitive inhibition for samples 997 and 395.

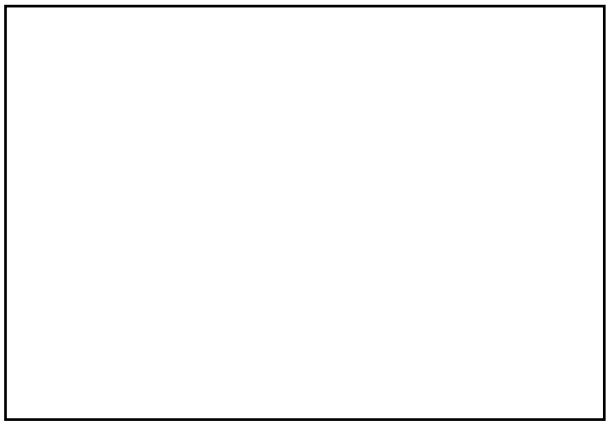


Table 3

Predominance of MAD20 over K1 alleles in P. falciparum isolates in Uganda.[a]

		MSPl alleles		
Region[b]	N	MAD20 (E-TSR)	Kl (Q-KNG)	Mixed (MAD20/K1)
Kampala (Central)	17	17 (17/17)	0 (0/17)	2 (2/17)
Kabarole (Western)	8	8 (8/8)	0 (0/8)	0 (0/8)
Apac (Northern)	8	8 (8/8)	0 (0/8)	1 (1/8)
Total	33	33 (33/33)	0 (0/8)	3 (3/33)
Percent		100	0	9

[a]Values are numbers of samples containing the MSPl allele with the frequency in parenthesis.
[b]Regions from which the genotyped *P. falciparum* isolates were collected.

The predominant allele of MSP1$_{19}$ in Uganda is E-TSR (MAD 20): The presence of nested PCR products of approximately 480 and 230 bp representing MAD20 and Kl alleles, respectively, were scored in 33 individual *P. falciparum* isolates from three different regions of Uganda, namely, Central (Kampala), Western (Kabarole) and Northern (Apac) regions (Table 3). All isolates contained parasites of the E-TSR (MAD20) allelic type. However three isolates were mixed infections in which the E-TSR (MAD20) and Q-KNG (K1) allelic types were also detected, thus demonstrating the paucity of the K1 allelic type in these isolates.

DISCUSSION

MSPl$_{19}$ is a promising *P. falciparum* blood stage vaccine currently under development(1). Previous studies have shown that antibodies to the carboxy terminal fragment of MSPl are associated with clinical immunity to malaria in West African children(13,14). The major IgG subclass response to MSPl$_{19}$ appears to be the opsonising IgGl and

IgG3 antibodies which predominate in the sera of clinically immune individuals and which appear to be responsible for both inhibition of merozoite invasion of erythrocytes and killing of intra-erythrocytic parasites by monocytes (Egan., A.E *et al* in press). We have undertaken studies of human IgG subclass responses to *P. falciparum* $MSPl_{19}$ in a region of intense malaria transmission in Uganda with the major goal of studying the role of anti-$MSPl_{19}$ antibodies in clinical immunity to malaria and to attempt to extend the findings reported in West African children to children in this part of Uganda. Our studies have confirmed that IgGl and IgG3 antibodies are the predominant IgG antibodies in residents of Atopi Parish and that the mean levels and prevalence of these antibodies increased with age. Interestingly, vigorous IgGl responses appeared earlier and were evident in children as early as five years. Whereas in the Gambian study there was a distinct paucity of IgG3 antibodies, in the Ugandan study there was a significant and vigorous response (mean OD values and prevalence) by IgG3 subclass although strong IgG3 responses were delayed and appeared only in children above seven to eight years. As with the Gambian population, we observed almost background levels of the IgG2 and IgG4 subclass responses in Ugandan subjects. Our current data has one major limitation. The samples were obtained from one time point in a cross-sectional survey. Thus, although yielding important insights about the possible roles of IgG1 and IgG3 antibodies in the host-parasite relationship during malaria infection in our study population, the important correlations between antibody levels on the one hand, and parasite densities or clinical immunity on the other hand, can not be studied.

We confirmed that the majority of our study population have cross-reactive antibodies against all $MSPl_{19}$ variants. This is in agreement with previous studies in West Africa(18) and augurs well for the development of a vaccine based on $MSP1_{19}$. However, we have identified a small minority (8.3 %) of subjects who recognise only the KNG but not the TSR variants of $MSPl_{19}$. The impact of this finding to the future application of $MSPl_{19}$ vaccine remains unclear. Previous work has reported the predominance of MAD20 allelic types and a paucity of K1 allelic types in African *P. falciparum* isolates(19, 20). We have confirmed the abundance of MAD20 and the rarity of K1 allelic types in *P. falciparum* isolates from Atopi Parish and other areas of Uganda.

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