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Differences in Anti Malarial Antibody Concentrations and Specificities between Male and Female Ugandan Children

Brenda Apio Okech[1,2*], Corran P[3], Todd J[3], Agwang C[2], Riley EM[4] and Egwang TG[2]

[1]UVRI-IAVI HIV Vaccine Program, Uganda

[2]Med Biotech Laboratories, Uganda

[3]London School of Hygiene and Tropical Medicine, UK

[4]Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, UK

Abstract

In Uganda malaria poses a formidable burden to an over-stretched and under-funded public health system trying to cope with other pressing health problems like HIV/AIDS and tuberculosis. Our earlier work on asexual blood stage malaria vaccine candidate merozoite surface protein (MSP119) provided a novel means of assessing antibody quality using altered proteins as well as ELISA competition assays with mouse monoclonal antibodies of known specificities. Our current hypothesis is that there is differential antibody level and quality between the sexes. This is based on earlier work in schistosomiasis showing higher levels of antibodies in females. Females have also been shown to have lower malaria parasite density although the explanation for this was thought to be hormonal.

In this study, levels of antibodies to several malaria antigens were measured in Ugandan school-going children in Northern Uganda, aged 7 to 16-years, to determine any difference in response between males and females. The qualities and specificities of MSP-1$_{19}$ antibodies in females and males were also determined by ELISA.

Overall females had higher levels and prevalence of antibodies to the merozoite proteins studied. These data suggest that dissection of the role of sex in naturally acquired immunity to malaria might identify drivers of the quality of the immune response. To our knowledge, this is the first report of higher levels of malaria antibodies to some asexual antigens in females compared to males in humans.

Introduction

Over one million deaths occur in Sub-Saharan Africa annually due to *Plasmodium falciparum* malaria (WHO, 2002). The deaths are a result of complications of malaria such as severe malaria-associated anaemia and cerebral malaria (WHO, 2000). In Uganda malaria poses a formidable burden to an over-stretched and under-funded public health system trying to cope with other pressing health problems like HIV/AIDS and tuberculosis. Twenty five to thirty percent of all out-patient visits and 300 infant deaths daily in Uganda are due to malaria alone, not to mention the significant proportion of low birth weight deliveries due to malaria during pregnancy [1,2]. A malaria vaccine that could interrupt the asexual blood stage in the host would prevent symptoms of malaria. A number of antigens have been proposed as vaccine candidates for this stage of the parasite. Some of the promising malaria vaccine candidates are serine rich antigen (SERA) [3], merozoite surface proteins 1, 2, 3 (MSP-1, MSP-2, MSP-3) [4-6], apical membrane protein 1 (AMA-1) [7], and ring infected erythrocyte surface antigen (RESA or Pf155) [8]. MSP-1 is proteolytically processed, post-translationally, in several stages. The primary processing step generates fragments of 83, 42, 38 and 30 kDa that persist on the surface of extracellular merozoites as a non-covalently linked complex held by the GPI anchor attached to the C-terminus of MSP-1$_{42}$ [9,10]. Secondary processing occurs during erythrocyte invasion and involves the cleavage of the membrane bound component of the 42 kDa complex at a single site to two smaller fragments of MSP-1$_{19}$ and MSP-1$_{33}$ [11,12]. Monoclonal antibodies to MSP-1$_{19}$ which inhibit MSP-1 secondary proteolytic processing also inhibit invasion [13]. Blocking monoclonal antibodies may interfere with the binding of inhibitory antibodies, allowing processing of MSP-1$_{42}$ and invasion of the erythrocyte to take place, while some are antibodies are neutral [14,15].

*Corresponding author: Brenda Apio Okech, UVRI-IAVI HIV Vaccine Program, Med Biotech Laboratories, Kampala, Uganda

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Our earlier work on asexual, blood stage malaria vaccine candidate merozoite surface protein (MSP-1$_{19}$), provided a novel means of assessing antibody quality using altered proteins as well as ELISA competition assays with mouse monoclonal antibodies of known specificities [3]. Our current hypothesis is that there is differential antibody level and quality between the sexes. This is based on earlier work in schistosomiasis showing higher levels of antibodies in females [16]. Females have also been shown to have lower malaria parasite density although the explanation for this was thought to be hormonal [17]. Sex-based differences in immune responses to malaria infection have been reported in mice with females producing higher concentrations of cytokines such as IL-10 as well as higher total IgG and IgG1 compared to males [18].

In this study, levels of antibodies to several malaria antigens were measured in Ugandan school-going children, aged 7 to 16-years, to determine differential responses between males and females. The quality and specificities of MSP-1$_{19}$ antibodies in females and males were also determined. This is the first report to our knowledge of higher antibody levels to malaria merozoite surface antigens in females as well as a differential quality of IgG responses between the sexes in children.

Materials and Methods

Study site

The study took place in Atopi Parish, Maruzi County, in Apac District in Northern Uganda. Atopi is located about 300 km from Kampala, the capital city of Uganda. The study site is located within a region of intense perennial malaria transmission whose malariometric indices have been described previously [19]. Apac District covers an area of 6,684 sq km and ranges in altitude between 1,350 and 1,500 metres above sea level. The average annual rainfall is 1,350 mm and the average temperature is 27 °C. Luo is the main language spoken in the district and 98% of the population is of the Langi ethnic group, making it a more or less homogeneous society. Approx. 80 percent of Apac's residents are engaged in subsistence farming. At the time of this study, the population was estimated at 676,244 according to the national census with a population density of 115 people per sq km. There are two major rainy seasons in this area, from March to May and then from July to August. And these are the peaks of malaria transmission. A study in 1995 showed that malaria transmission in Atopi Parish is hyper-endemic, and that acquired anti-parasite immunity appears by approximately seven years of age [19]. *Plasmodium falciparum* is the major malaria parasite in this area.

Study design and study population

The design was a longitudinal, prospective cohort study of children attending Atopi Primary School in Atopi Parish. Briefly, ethical approval was obtained from the Uganda National Council for Science and Technology and parental consent was obtained prior to the enrolment of the children. A standard questionnaire, including recent clinical history and the use of anti-malarial drugs and protective anti-mosquito measures,

was completed followed by an examination by a physician. At the initial visit, 183 children were seen, treated with a full curative dose of Fansidar® to remove any prior malaria infection, and a whole blood (2 mls) sample was collected by finger prick using EDTA-containing vacutainers. Thin and thick blood smears were made on labelled glass slides. Serum was separated from cell pellets by centrifugation and both sera and cell pellets stored at -80 °C. The 159 children who had cleared all parasites from their blood by 2 weeks after Fansidar treatment were recruited. The target age of children who were recruited was 7 to 16-years. Out of the 159 children, 3 were excluded from all analyses because their age was out of range. A total of 156 children were therefore included in the study. The recruited children were followed-up fortnightly to assess reinfection and any associated morbidity. At each of the subsequent visits blood slides for malaria microscopy were made and the children examined and treated as appropriate. The study was carried out in year 2000.

Microscopy

Thick and thin blood smears for each child were prepared, air dried and Giemsa-stained for microscopic confirmation of *P. falciparum* parasites. A rapid examination of thick films was carried out in Apac so that children with confirmed *P. falciparum* infections received prompt treatment with antimalarial drugs according to the national guidelines. Thin films were examined in detail in Kampala to identify the different species of malaria parasites while thick films were used for the enumeration of parasites. The parasite density per µl of blood was calculated from the number of parasites per 200 white blood cells (WBC), assuming an average normal WBC count of 8000/µL. A blood slide was declared negative after reading 100 high power fields.

Sickle cell genotyping

Sickle cell status of all the children was assessed through sickle cell hemoglobin electrophoresis. The children's blood was centrifuged and the red cell pellets washed in saline and stored at -20 °C until ready for use. Hemolyzed patient samples and the haemoglobin controls (HbAA (normal adult blood), HbAS (blood from a subject with the sickle cell trait), HbAF (blood from an infant below 3 months), and HbAC (blood from a person with an HbAC trait)) supplied by Helena Biosciences (Sunderland, UK) were resolved by electrophoresis on cellulose acetate plates at 350 V for 25 minutes in alkaline buffer (pH 8.4-8.6) and stained with Ponceau S for 5 minutes. The plates were de-stained, dried, and stored for permanent record keeping. The hemoglobin types were identified by comparing the migration of bands in the unknown specimens with the hemoglobin controls.

Enzyme linked immunosorbent assay (ELISA)

The following antigens were used in the study: Merozoite surface proteins MSP-1$_{19}$ (K1 sequence), MSP-1$_{42}$ (3D7 sequence), MSP-2 (FC27 sequence) and MSP-2 (3D7 sequence), SE36, SE50A, AMA-1, RESA and whole *P. falciparum* schizont lysate.

The *P. falciparum* schizont lysate was made by rupturing

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mature percoll separated schizonts by repeated freezing and thawing using dry ice and water at 37 °C. All other antigens are recombinant constructs expressed in *Escherichia coli*. Recombinant MSP-1$_{19}$ was prepared by standard techniques as a glutathione S-transferase (GST) fusion protein [20].

The quality of MSP-1$_{19}$ antibodies was measured using indirect ELISA with recombinant MSP-1$_{19}$ proteins in which specific residues had been mutated to destroy known targets of known non-protective (i.e. blocking or neutral) monoclonal antibodies [3,21]. These mutated MSP-1$_{19}$ antigens were also prepared as GST fusion proteins (as described by [21]. Altered protein M3 carries three amino acid substitutions-Glu to Tyr at residue 27, Leu to Arg at residue 31, and Glu to Leu at residue 43. The other two proteins carry these three substitutions and one additional substitution-Asn to Arg at residue 15 (M4X15) or Tyr to Ser at residue 34 (M4X34). The MSP antigens and GPI were donated by Anthony Holder. The SE50A and SE36 antigens were donated by Prof Toshihiro Horii.

Synthetic genes encoding SE47' and SE50A representing the amino terminus and central domains, respectively, of SERA5 were constructed using *E. coli* codons and the recombinant proteins were expressed in *E. coli* [22]. The synthetic gene construct encoding the recombinant antigen SE36 was identical to that encoding SE47 except that the 105-bp nucleotide sequence encoding a run of 35 serines were removed. The resulting SE36 is easier to purify and re-fold at a higher yield as described by [3].

The optimal antigen concentration for direct ELISA (1.0 μg/ml for all recombinant antigens and 1:1000 dilution for *P. falciparum* schizont lysate) were determined by checkerboard titration. All secondary antibodies used were conjugated to horseradish peroxidase (Dako Ltd, High Wycombe, UK). For the indirect ELISAs, rabbit anti-human IgG was used at a dilution of 1:5000. The plates used in all enzyme linked immunosorbent assays were flat-bottomed 96-well plates from Thermo Lab Systems (Immunolon® 4 plates, Dynatech, UK).

Indirect ELISA

The plates were coated overnight at 4 °C with 100 μl of antigen at a concentration of 1 μg/ml in carbonate coating buffer of pH 9.6. The plates were then washed 3 times in PBS/Tween 20 and blocked with 1% bovine serum albumin (BSA) or 1% skimmed milk powder in PBS/T, 150 μl per well for 3 h or at room temperature. The plates were again washed 3 times with PBS/Tween 20. The test sera were added (100 μl per well) at dilutions of 1:1000 in 1% BSA in PBS/T and the plates incubated overnight at 4 °C. After the plates were washed 4 times in PBS/Tween 20, 100 μl of horseradish peroxidase conjugated anti-human IgG diluted at 1:1000 in PBS/T was added per well and incubated at 37 °C for 1 hour. The plates were washed 4 times, o-phenylenediamine (OPD) substrate (Sigma) was added and the plates were developed for 10 minutes. The reaction was stopped with 25 μl of 2M sulphuric acid and the plates read at 492 nm.

Competition ELISA

The quality of MSP-1$_{19}$ antibodies was also measured using competition ELISA with mouse monoclonal antibodies

(MAbs) of known specificity [3]. MAbs 12.10 and 12.8 inhibit processing of MSP-1$_{42}$ to MSP-1$_{33}$ and MSP-1$_{19,}$ and thus prevent erythrocyte invasion leading to protection from malaria, whereas MAbs 1E1 and 7.5 sterically interfere with binding of the inhibitory antibodies thereby blocking their function and permitting merozite invasion; MAbs 2F10 and 8A12 are neutral and have no effect on processing or invasion. MAbs were titrated by a direct ELISA for binding to wild-type MSP-1$_{19}$ antigens; each MAb was used in competition assays at a concentration that gave an OD just below the maximal OD for that antibody (i.e., just below the top of the linear part of the titration curve), such that any reduction in OD for the binding of the MAb was linearly associated with the amount of antibody bound. Microtiter plates were coated overnight with native or mutant MSP-1$_{19}$ (0.1 μg/ml) followed by washing. Serum at a dilution of 1:50 or 1:250 was added to duplicate wells, and the plates incubated overnight at 4 °C. After washing, a fixed amount of mouse MAb was added, and the plates were incubated again overnight at 4 °C. Rabbit anti-mouse immunoglobulins were used at a dilution 1:1000. The plates were washed, and binding of the MAb was detected with o-phenylenediamine and the reaction stopped with sulphuric acid and read as above. Sera were classified as being able to reduce MAb binding to MSP-1$_{19}$ by ≥ 50% at a dilution of 1:50 or 1:250 or as being non-competitive (< 50% inhibition of MAb binding at a dilution of 1:50).

Statistical Analysis

EPI Info 6 program (CDC Atlanta, Epidemiology Program Office, Atlanta, GA) was used for the entry of the malariometric and laboratory data into the computer. All statistical analysis was carried out using STATA 8.1 (Statacorp, USA, 2004). The differences in mean serum levels of antibody in between male and female children were assessed using a non-parametric statistical method, Wilcoxon rank-sum (Mann-Whitney) test. Differences and associations of antibody levels and prevalence of positive antibodies by sex were assessed using chi-squared tests.

Results

Comparisons of antibody responses by sex

There were no significant differences between the sexes in age, village of residence, bed net usage, maximum malarial parasitemia, number of clinical episodes or antimalarial drug use over the study period (data not shown). However, there was a statistically significant difference between females and males in median antibody binding (expressed as optical density, OD) to some of the malaria antigens as shown in (Table 1). Antibody OD to MSP-1$_{42}$, MSP-1$_{19,}$ MSP-2 FC27, MSP-2 3D7 but not RESA, AMA-1, and SE50A, were significantly higher in females than males. On the other hand, antibody levels to *P. falciparum* whole parasite lysate and SE36 antigens were significantly higher in males than females.

Differences in fine specificities of the MSP119 response by sex

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Table 1: Comparison of antibody levels (optical density) to asexual blood stage antigens according to sex.

Antigen	Females		Males		P value
	N	Median (IQR)	N	Median (IQR)	(Mann-Whitney U)
MSP-1$_{19}$	84	1.307 (0.226-2.317)	72	0.227 (0.029-1.133)	**0.0001**
MSP-1$_{42}$	84	1.681 (0.774-2.348)	72	0.936 (0.503-1.633)	**0.0014**
MSP-2 3D7	84	1.888 (0.989-2.475)	72	1.539 (0.530-2.357)	**0.038**
MSP-2 FC27	84	2.316 (1.573-2.592)	72	1.726 (0.945-2.549)	**0.029**
AMA-1	82	0.292 (0.155-0.406)	71	0.314 (0.213-0.403)	0.546
Pf Lysate	82	0.149 (0.101-0.214)	71	0.191 (0.102-0.246)	0.065
RESA	67	0.302 (0.405-0.667)	60	0.272 (0.370-0.649)	0.589
SE50A	82	0.010 (0.004-0.017)	82	0.007 (0.004-0.014)	0.35
SE36	84	0.207 (0.214-0.243)	72	0.205 (0.213-0.261)	0.37

Table 2: Comparison of the specificity of anti-MSP-1$_{19}$ antibodies between females and males, defined as the proportion of serum samples (at a dilution of 1:250) able to inhibit the binding of mouse monoclonal antibodies (of known binding specificities) to MSP-1$_{19}$ in a competition ELISA.

Mab	Reduction in Mab binding (%)		
	SEX		
	Female N = 84 n (%)	Male N = 72 n (%)	χ^2 (П)
12.1	41 (49)	17 (23)	10.54 (0.001)
12.8	35 (42)	19 (26)	4.00 (0.046)
1.00E + 01	37 (44)	18 (25)	6.16 (0.013)
7.5	32 (38)	15 (21)	5.49 (0.019)
8 A 12	33 (39)	15 (21)	6.20 (0.013)
2F10	30 (36)	13 (18)	6.05 (0.014)

The most significant difference between the sexes was in antibody OD to MSP-1$_{19}$. Antibodies to MSP-1$_{19}$ were assessed further by comparing the distribution of antibody specificities. The specificities were assayed by competition ELISA using mouse monoclonal antibodies of known function, i.e. inhibition of MSP-1 processing and invasion (MAb 12.10 and 12.8), blocking of the inhibitory antibodies (MAb 1E1 and 7.5) or neutral (no effect on MSP-1 processing; 8A12 and 2F10). A higher proportion of sera from females could compete with the different mouse monoclonal antibodies compared to males (Table 2). Antibody fine specificities were also measured by direct ELISA with mutated MSP-1$_{19}$ proteins designed to prevent binding of putative 'non-protective' antibodies. Neither age, village of residence, bed net use nor sickle cell status significantly affected the prevalence of the putative inhibitory antibodies but antibodies from females were significantly more likely to recognize altered MSP-1$_{19}$ proteins (with two or more mutations) than were antibodies from their male counterparts (59% for females compared to 25% for males, P < 0.001; (Table 3). Taken together our data reveal differences in antibody concentrations, specificity (and putative inhibitory capacity) between male and female chil-

dren aged 7-16-years in Atopi parish in Apac district.

Discussion

The major finding of this study was that female primary school-aged children had higher levels and higher prevalences of antibodies to malarial merozoite proteins studied than male children. There were no obvious differences in age or other demographic parameters that might have confounded this association. In Uganda survival is higher in females under 5-years [23]. Whilst there are behavioural explanations for sex or gender related differences in exposure to parasitic infection in some specific situations [24-26] there is considerable evidence that sex-determined differences in the strength of the immune response after exposure are substantially influenced by sex hormones.

There are many examples of sex-associated differences in immune responses [27,28] where behavioural differences can be ruled out, leaving the influence of sex hormones on the immune system as a likely driver of sex associated difference [29,30]. Administration of both measles and Diphtheria-Tetanus-Pertussis vaccines is associated with higher antibody response in females than males and has been linked to differences in the innate immune response, specifically Toll-like receptor 4 (TLR4) signalling [31] and a study of school children by found higher levels of TNF-α in non-obese females compared to their non-obese male counterparts [32]. However, these differences were seen in pre-pubertal school children as well as older students suggesting that factors other than hormonal differences may be at play.

With respect to malaria, Thai women vaccinated with the candidate SP f66 malaria vaccine higher antibody levels than men but also had more frequent adverse effects of vaccination [33], parasite densities are reportedly lower in females than males in endemic areas [17] and in males with higher dehydroepiandrosterone sulphate hormone levels [34]. Frequencies of cord blood regulatory T cell differed by infant sex, with significantly lower frequencies in female than in male neonates who were exposed to malaria in utero [35]. Studies in mice suggest that elevated testosterone concentrations in males reduce adaptive immunity and contribute to sex dif-

Table 3: Comparison of the specificity of anti-MSP1-$_{19}$ antibodies (defined as the proportion of sera binding to variant antigens representing putative 'inhibitory' specificities) by sex, age, village of residence, bed net use and sickle cell status.

		Prevalence of 'inhibitory' phenotype antibodies								
		M3X (N = 52)			M4X34 (N = 52)			M4X15 (N = 52)		
		n (%)	c^2	P	n (%)	c^2	P	n (%)	c^2	P
Sex	M (N = 52)	13 (25)	13.71	**< 0.001**	13 (25)	13.71	**< 0.001**	13 (25)	13.71	**< 0.001**
	F (N = 66)	39 (59)			39 (59)			39 (59)		
Age (yrs)	7-9 (N = 19)	19 (12)	2.88	0.3	6 (12)	2.88	0.3	6 (12)	2.88	0.3
	10-12 (N = 64)	27 (52)			27 (52)			27 (52)		
	13-16 (N = 35)	19 (37)			19 (37)			19 (37)		
Village Other	(N = 23)	7 (30)	3.52	0.3	7 (30)	3.52	0.3	7 (30)	3.52	0.3
Atopi	(N = 65)	30 (46)			30 (46)			30 (46)		
Ayera	(N = 13)	8 (61)			8 (61)			8 (61)		
Baraboce	(N = 17)	7 (41)			7 (41)			7 (41)		
Normal spleen	(N = 98)	41 (42)	1.17	0.3	41 (42)	1.17	0.3	41 (42)	1.17	0.3
Enlarged spleen	(N = 20)	11 (55)			11 (55)			11 (55)		
	HbAA (N = 164)	36 (43)	0	1	36 (43)	0	1	36 (43)	0	1
	HbAS (N = 59)	14 (44)			14 (44)			14 (44)		
Bed net use	No (N = 101)	45 (45)	0.07	0.8	45 (45)	0.07	0.8	45 (45)	0.07	0.8
	Yes (N = 17)	7 (41)			7 (41)			7 (41)		

Apart from village (df = 3) all tests were for 1 degree of freedom. All values in **bold** represent statistically significant differences i.e. P < 0.05. (HbAA = non-sickle cell trait; HbAS = children with sickle cell trait).

ferences in malaria vaccine efficacy [36]. Similar observations have been noted for schistosomiasis [16]. Importantly, in this study, females had a higher prevalence of antibodies to specific variants of the major merozoite protein MSP-1 that may be indicative of protective immunity. However, our study did not look at rates of reinfection or clinical malarial disease and so we cannot infer that the higher prevalence of antibodies confers protection. Clinical and parasitological studies have shown that males are more susceptible to infection or disease [37,38]. Our study is in agreement with a previous study that did not show differences in sickle cell status between males and females [39].

One limitation of this work is that there was no information on the HIV status of the subjects. HIV is a possible confounder in studies of immune response as the virus attacks the CD4+ T cells that are important in providing help to B cells so that they can proliferate and produce antibodies [40,41]. Although adult HIV rates in Uganda at the time of the study were approximately 8% (US Census Bureau database, June 2002), HIV rates are usually highest in individuals from 17-35-years and in children under 2-years born to HIV positive mothers and we expect therefore that very few of our study children were likely to be HIV positive. Moreover, as antiretroviral therapy was not widely available in this area at the time of the study, any study children who had been infected at birth would very likely have been symptomatic. Thus, considering the low prevalence of HIV in rural areas and specifically in this age group, we believe that HIV is not expected to confound antibody responses in this study.

In summary, we have observed higher antibody levels and differences in antibody specificities for major merozoite antigens, some of which remain candidates for a malaria vaccine, between males and females. These observations may have implications for the development and deployment of malaria vaccines if they translate into sex-related differences in the response to malaria vaccination. Attention should be given to analysis of outcome of malaria vaccine studies by sex and future studies should be sufficiently powered to allow robust analysis of sex differences.

Acknowledgement

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