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Fine Specificity of Serum Antibodies to *Plasmodium falciparum* Merozoite Surface Protein, PfMSP-1_{19}, Predicts Protection from Malaria Infection and High-Density Parasitemia†

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Antibodies to the C terminus of the *Plasmodium falciparum* merozoite surface protein, PfMSP-1_{19}, may inhibit merozoite invasion or block the effects of inhibitory antibodies. Here, using a competition enzyme-linked immunosorbent assay and antibody binding to wild-type and mutated recombinant proteins, we show that there are marked variations between individuals in the fine specificity of naturally acquired anti-MSP-1_{19} antibodies. Furthermore, although neither the prevalence nor the concentration of total anti-MSP-1_{19} antibodies was associated with resistance to malaria in African children, significant associations were observed between antibody fine specificity and subsequent risk of infection and high-density parasitemia during a follow-up period. Thus, the fine specificity of naturally acquired human anti-MSP-1_{19} antibodies is crucial in determining their function. Future field studies, including the evaluation of PfMSP-1 vaccine trials, should include assays that explore antibody fine specificity as well as titer.

Plasmodium falciparum merozoite surface protein (PfMSP-1) is synthesized as a ca. 200-kDa precursor protein which is cleaved, at the time of merozoite release from the infected red blood cell, into four polypeptides (MSP-1_{83}, MSP-1_{30}, MSP-1_{38}, and MSP-1_{42}) that remain on the merozoite surface as a glycosylphosphatidylinositol (GPI)-anchored complex. During erythrocyte invasion, a second cleavage occurs, and the entire complex, except for the GPI-anchored C-terminal PfMSP-1_{19} fragment, is shed. Inhibition of this final processing step prevents erythrocyte invasion (reviewed in reference 14).

In both mice and monkeys, immunization with MSP-1_{19} or MSP-1_{42} protects against challenge infection (4, 11, 17). Protection is antibody dependent (11, 12, 27) and independent of the Fcγ receptor (25, 27). A substantial proportion of the invasion-inhibiting activity in human immune serum is associated with antibodies to MSP-1_{19} (23), and affinity-purified MSP-1_{19}-specific IgG from human serum inhibits erythrocyte invasion in vitro (6). Antibodies act, in part, by inhibiting the final stage of MSP-1_{19} processing (1, 10, 26), although correlations between protection and inhibition of processing in PfMSP-1_{19}-immunized monkeys are not absolute (7), suggesting that other mechanisms contribute to protection. Whatever the mechanism, the fine specificity of the antibodies is crucial for their ability to inhibit merozoite invasion. Anti-MSP-1_{19} antibodies with overlapping specificities (26) can compete with processing-inhibiting antibodies without themselves inhibiting processing, thus blocking the protective effect. Other anti-MSP-1_{19} antibodies are "neutral"—they appear to have no effect on processing and do not block processing-inhibiting antibodies.

Studies of populations naturally exposed to *P. falciparum* have shown various degrees of association between anti-MSP-1_{19} antibodies and protection from clinical malaria (2, 5, 9, 13, 16), and it was recently shown that there is no correlation between naturally acquired human anti-MSP-1_{19} antibody titers and inhibition of MSP-1_{19} processing (22). We suggest that protection is associated with the presence of antibodies to certain epitopes; antibodies that bind to MSP-1_{19} with a different fine specificity may be unable to protect and may block protective antibodies. Thus, the fine specificity of anti-MSP-1_{19} antibodies rather than their simple prevalence or titer may be a better predictor of their protective efficacy.

We have tested this hypothesis by investigating the fine specificity of anti-MSP-1_{19} antibodies in sera from children in areas where malaria is endemic in The Gambia and Uganda. We have determined the ability of individual sera to compete for binding to recombinant MSP-1_{19} (rMSP-1_{19}) by using a panel of monoclonal antibodies (MAbs) with known invasion-inhibiting, blocking, or neutral function. We have also tested sera for binding to rMSP-1_{19} mutants in which epitopes recognized by blocking MAbs have been disrupted. Finally, we have

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† This article is dedicated to the memory of Steve Bennett, who died in March 2003 at the age of 52 years.

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TABLE 1. Prevalence of antibodies to rMSP-1_{19}, their ability to compete with MAbs, and association with age and morbidity for Gambian children

Parameter	n^a	rMSP1_{19} (n = 247)	No. (%) of the following antibodies that competed at the indicated dilution[b]:											
			12.8 (n = 237)		12.10 (n = 243)		1E1 (n = 241)		7.5 (n = 241)		8A12 (n = 241)		2F10 (n = 237)	
			1:50	1:250	1:50	1:250	1:50	1:250	1:50	1:250	1:50	1:250	1:50	1:250
Age group (yr)														
2–4	74	16 (23)	3 (4)	4 (6)	7 (10)	6 (8)	1 (1)	1 (1)	4 (6)	4 (6)	5 (7)	4 (6)	3 (4)	1 (1)
5–6	92	34 (40)	7 (9)	10 (12)	16 (19)	8 (10)	8 (10)	6 (7)	8 (10)	4 (5)	7 (8)	6 (7)	5 (6)	5 (6)
7–9	94	33 (36)	7 (8)	8 (9)	9 (10)	10 (11)	7 (8)	7 (8)	11 (12)	3 (3)	9 (10)	7 (8)	6 (7)	2 (2)
Total	260	83 (34)	17 (7)	22 (9)	32 (13)	24 (10)	16 (7)	14 (6)	23 (10)	11 (5)	21 (9)	17 (7)	14 (6)	8 (3)
χ^2 (P)[c,d]		2.95 (0.09)	1.11 (0.29)		0.15 (0.69)		5.58 (0.018)		0.63 (0.43)		0.83 (0.36)		0.43 (0.51)	
Clinical category														
No infection	55	19 (35)	5 (9)	6 (11)	9 (16)	6 (11)	8 (14)	3 (5)	6 (11)	0 (0)	5 (9)	2 (4)	2 (4)	2 (4)
Asymptomatic infection	58	14 (27)	4 (8)	4 (8)	7 (13)	4 (8)	3 (6)	4 (8)	4 (8)	3 (6)	2 (4)	3 (6)	1 (2)	1 (2)
Clinical malaria	87	26 (33)	4 (5)	7 (9)	8 (10)	8 (10)	3 (4)	3 (4)	8 (11)	5 (7)	7 (9)	6 (8)	4 (5)	3 (4)
Fever plus low level of parasitemia	29	8 (30)	2 (8)	2 (8)	2 (7)	2 (7)	1 (4)	1 (4)	3 (11)	0 (0)	3 (11)	3 (11)	2 (7)	1 (4)
Total		67 (31)	15 (7)	19 (9)	26 (12)	20 (9)	15 (7)	11 (5)	21 (10)	8 (4)	17 (8)	14 (7)	9 (4)	7 (3)
χ^2 (P)[c,e]		0.43 (0.51)	0.97 (0.32)		1.35 (0.24)		4.06 (0.04)		0.50 (0.48)		0.23 (0.63)		0.01 (0.9)	
Age-corrected χ^2 (P)[c]		0.68 (0.41)	1.26 (0.26)		1.45 (0.23)		4.99 (0.03)		0.45 (0.50)		0.16 (0.69)		0.01 (0.9)	

[a] Maximum number of samples available, however, due to limited sample volumes, not all of the samples could be tested for all of the antibodies. Thus, the denominators for the calculation of percentages varied slightly between antibodies.

[b] For 1:50, data are for sera that competed at a dilution of 1:50 but not 1:250. For 1:250, data are for sera that competed at a dilution of 1:250.

[c] Comparison of sera that competed at either dilution (1:50 or 1:250) with sera that did not compete. There was 1 df for the χ^2 test.

[d] Trend across age groups.

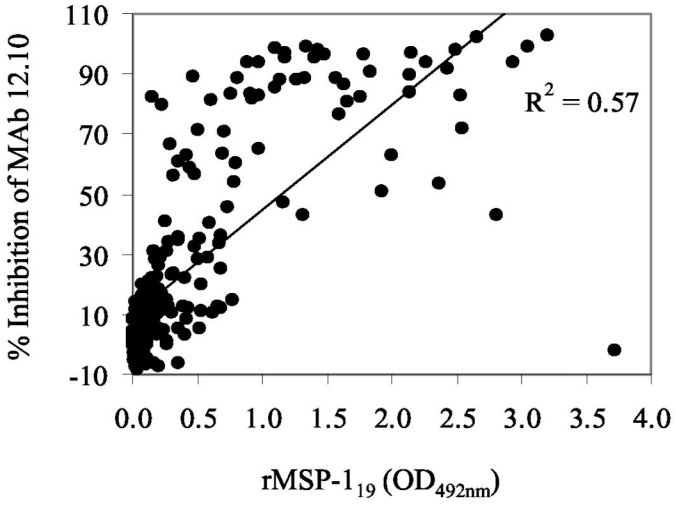
[e] Comparison of no infection with any infection.

TABLE 2. Prevalence of antibodies to rMSP-1_{19}, their ability to compete with MAbs, and association with age and subsequent parasitemia for Ugandan children m

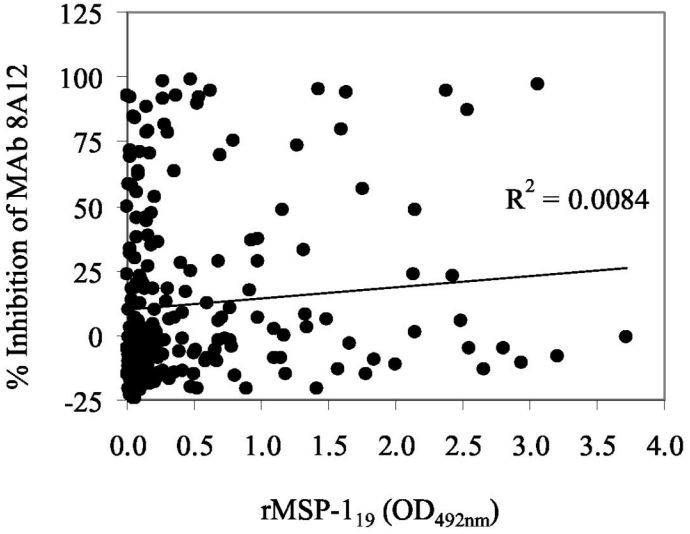
Parameter	*n*	rMSP1_{19}	No. (%) of the following antibodies that gave an OD of >0.263 at the indicated dilution:																	
			12.8			12.10			1E1			7.5			8A12[a]			2F10[a]		
			1:50	1:250	Combined	1:50	1:250	Combined	1:50	1:250	Combined	1:50	1:250	Combined	1:50	1:250	Combined	1:50	1:250	Combined
Total	156	94 (60)	41 (26)	54 (35)	95 (61)	27 (17)	58 (37)	85 (54)	36 (23)	55 (35)	91 (58)	34 (22)	47 (30)	81 (52)	30 (20)	48 (32)	78 (52)	24 (16)	43 (28)	67 (44)
Age group (yr)																				
7–9	27	16 (59)	11 (41)	6 (22)		6 (22)	7 (26)		5 (19)	10 (37)		7 (26)	8 (30)		7 (27)	7 (27)		3 (12)	7 (27)	
10–12	85	49 (58)	16 (19)	32 (38)		10 (12)	34 (40)		18 (21)	30 (35)		17 (20)	23 (27)		13 (15)	26 (31)		13 (15)	22 (26)	
13–16	44	29 (66)	14 (32)	16 (36)		11 (25)	17 (39)		13 (30)	15 (34)		10 (23)	16 (36)		10 (24)	15 (37)		8 (20)	14 (34)	
χ^2 (*P*)[b]		0.89 (0.34)	0.43 (0.51)			1.92 (0.17)			0.57 (0.45)			0.27 (0.60)			0.65 (0.42)			1.77 (0.18)		
Maximum parasitemia[c]																				
<1,000	58	40 (69)	17 (29)	21 (36)		8 (14)	28 (28)		10 (17)	27 (47)		13 (22)	22 (38)		10 (17)	25 (44)		12 (21)	22 (39)	
1,000–5,000	70	38 (54)	16 (23)	25 (36)		9 (13)	24 (34)		17 (24)	22 (31)		13 (19)	19 (27)		13 (19)	19 (28)		5 (7)	18 (26)	
>5,000	28	16 (57)	8 (29)	8 (29)		10 (36)	6 (21)		9 (32)	6 (21)		8 (29)	6 (21)		7 (28)	4 (16)		7 (28)	3 (12)	
χ^2 (*P*)[d]		1.81 (0.18)	0.73 (0.39)			0.67 (0.42)			1.04 (0.31)			1.47 (0.23)			2.97 (0.08)			5.17 (0.023)		
χ^2 (*P*)[e]			0.38 (0.54)			6.28 (0.012)			5.93 (0.015)			2.87 (0.09)			7.19 (0.007)			6.38 (0.012)		

[a] Data for 8A12 and 2F10 were obtained only for 151 children.
[b] Trend across age groups (1 df) for a comparison of sera that competed at either dilution (1:50 or 1:250) with sera that did not compete.
[c] Parasites per microliter over 14 follow-up visits.
[d] Trend across parasitemia groups (1 df) for a comparison of sera that competed at either dilution (1:50 or 1:250) with sera that did not compete.
[e] Trend across parasitemia groups (1 df) for a comparison of sera that competed at a dilution of 1:250 with sera that competed at 1:50 or did not compete.

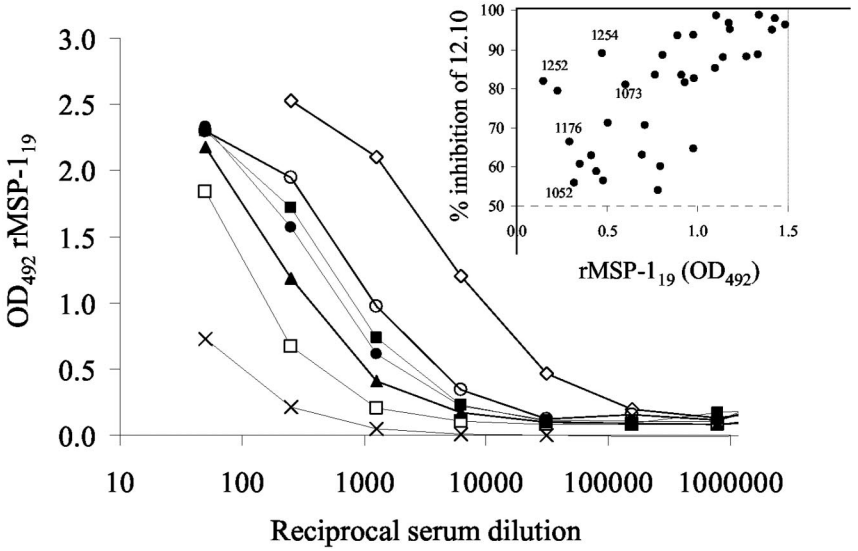
a


% Inhibition of MAb 12.10
110
90
70
50
30
10
-10
$R^2 = 0.57$
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0
rMSP-1_{19} (OD_{492nm})

b


% Inhibition of MAb 8A12
125
100
75
50
25
0
-25
$R^2 = 0.0084$
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0
rMSP-1_{19} (OD_{492nm})

c


OD_{492} rMSP-1_{19}
3.0
2.5
2.0
1.5
1.0
0.5
0.0
10 100 1000 10000 100000 1000000
Reciprocal serum dilution
% inhibition of 12.10
100
90
80
70
60
50
1254
1252
1073
1176
1052
0.0 0.5 1.0 1.5
rMSP-1_{19} (OD_{492})

sought associations between the fine specificity of the antibody response and resistance to a high parasite load or clinical malaria.

MATERIALS AND METHODS

Study populations. The Gambian study was described in detail previously (24). Antibodies in serum collected prior to the annual rainy season were assayed, and children were monitored once every 2 weeks for 5 months for symptoms of malaria, including fever. Children were classified as having no evidence of infection, clinical malaria (at least one episode of a temperature of ≥37.5°C and parasitemia of ≥5,000 parasites/μl), fever with a low level of parasitemia (a temperature of ≥37.5°C but parasitemia of ≤5,000 parasites/μl), or asymptomatic infection (parasitemia or acquired splenomegaly without fever).

The Ugandan children (aged 7 to 16 years) were from Apac, northern Uganda; *P. falciparum* prevalence prior to the study was >80%. All children received a single dose of pyrimethamine-sulfadoxine (Fansidar), and those who were blood film negative 2 weeks later were monitored by morbidity surveillance blood film analysis every 2 weeks for 5 months during the high-transmission season. Clinical malaria was treated with Fansidar. Sixty percent of the children became reinfected within 1 month, and most were parasitemic much of the time (average parasite prevalence for the duration of the follow-up period, 58%). Susceptibility to malaria thus was assessed by comparing the maximum observed parasite densities during the follow-up period.

Informed consent was obtained from all volunteers, and ethical approval was obtained from the ethical review committee of the London School of Hygiene and Tropical Medicine, the Medical Research Council-Gambia Government ethical review committee, and the Ugandan Ministry of Health.

Antigens. rMSP-1_{19} was prepared by standard techniques as a glutathione *S*-transferase (GST) fusion protein (3) and represents the Wellcome sequence of MSP-1_{19} (15, 19). Mutated rMSP-1_{19} antigens were also prepared as GST fusion proteins as described previously (26). Mutant M3 carries three amino acid substitutions—Glu to Tyr at reside 27, Leu to Arg at residue 31, and Glu to Leu at residue 43. The other two proteins carry these same three substitutions plus one additional substitution—Asn to Arg at residue 15 (M4X15) or Tyr to Ser at residue 34 (M4X34). Amino acid residues are numbered from the start of MSP-1_{19}.

MAbs. Inhibitory MAbs 12.10 and 12.8 (18) inhibit the secondary processing of MSP-1_{42} to MSP-1_{33} and MSP-1_{19} and the invasion of erythrocytes by merozoites (10). Both MAbs recognize a loop defined by residues 22 to 30 in the first epidermal growth factor-like domain (26) and bind mutually exclusively. Both MAbs compete with MAbs 1E1 and 7.5.

Blocking MAbs 1E1 (1) and 7.5 (18) inhibit neither invasion nor the processing of MSP-1_{42} (10) but compete with each other and with MAbs 12.10 and 12.8 and thus block their processing-inhibiting activity.

Neutral MAbs 2F10 and 8A12 (1, 10) have no effect on processing or invasion and do not block the functions of MAbs 12.8 and 12.10 (10).

ELISA. An indirect enzyme-linked immunosorbent assay (ELISA) was carried out as previously described (8) with 0.5 μg of wild-type or mutated rMSP-1_{19}–GST fusion protein/ml to coat plates and immune sera at a dilution of 1:1,000. Mean optical densities (ODs) were corrected for the binding of IgG to GST alone.

Competition ELISA. MAbs were titrated by a direct ELISA for binding to wild-type and mutated rMSP-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 0.1 μg of rMSP-1_{19} or mutated protein/ml, serum at a dilution of 1:50 or 1:250 was added to duplicate wells, and the plates were incubated overnight at 4°C. After washing was done, a fixed amount of mouse MAb was added, and the plates were incubated again overnight at 4°C. The plates were washed, and binding of the MAb was detected with rabbit anti-mouse IgG–horseradish peroxidase (Dako) and *o*-phenylenediamine. Sera were classified as being able to reduce MAb binding to rMSP-1_{19} by ≥50% at a dilution of 1:50 or 1:250 or as being noncompetitive (<50% inhibition of MAb binding at a dilution of 1:50).

Data analysis. Age was coded into categories: for The Gambia, 2 to 4 years, 5 to 6 years, and 7 to 9 years; and for Uganda, 7 to 9 years, 10 to 12 years, and 13 to 16 years. For the Gambian study, infection with malaria was based on the four classifications made over the season, and the final analysis compared children with any evidence of infection to those who had no infection. For the Ugandan study, peak parasite density detected during follow-up was classified as <1,000 asexual blood-stage parasites/μl, 1,000 to 4,999 parasites/μl, and >5,000 parasites/μl.

For each MAb, sera were classified as being moderately competitive (i.e., able to reduce MAb binding to rMSP-1_{19} by ≥50% at a dilution of 1:50), highly competitive (≥50% inhibition of MAb binding at a dilution of 1:250), or noncompetitive (<50% inhibition of MAb binding at a dilution of 1:50). For each MAb, statistical tests compared all competitive sera (at a dilution of either 1:50 or 1:250) to noncompetitive sera. The association between the ability of sera to compete and age or infection status was tested by using the χ^2 distribution with 1 df for a linear trend across age groups and infection categories. Logistic regression was used to assess the association between MAb competition and other explanatory factors after adjustment for age.

In total, four comparisons were made for inhibition of the binding of each of the six MAbs, with age and clinical morbidity being considered in the Gambian study and age and parasitemia being considered in the Ugandan study. To allow for the effect of multiple comparisons, results should be considered significant at the 5% level if the nominal *P* value is less than 0.002 (0.05/24).

RESULTS

Antibodies to rMSP-1_{19}. Anti-MSP-1_{19} OD values were unimodally distributed in the Gambian population, with 38% of sera giving OD values above the normal range for European control sera (Table 1), but we found no significant association between mean anti-MSP-1_{19} OD values and age or clinical outcome. These results confirm the results of an earlier study with this same set of sera, where an association with protection was found for a set of sera from Sierra Leone but not for Gambian sera (9). The frequency distribution of anti-MSP-1_{19} OD values was bimodal in the Ugandan cohort, with low responders giving OD values below 1.3 and high responders giving OD values above 1.3. The overall prevalence of anti-MSP-1_{19} antibodies (OD values above the normal range for European sera) was 60% and, again, the prevalence of antibodies did not increase significantly with age and did not differ between groups with different clinical outcomes (maximum parasitemia during follow up) (Table 2).

Human serum antibodies compete with MAbs for binding to rMSP-1_{19}. In the Gambian cohort, 56 of 243 sera (23%) inhibited the binding of MAb 12.10 (32 sera at a 1:50 dilution and 24 sera at a 1:250 dilution), but only 22 of 237 (9%) inhibited the binding of MAb 2F10 (14 sera at 1:50 and 8 sera at 1:250). Very few sera (3 to 10%) were able to compete with MAbs at a 1:250 dilution (Table 1).

The inhibition of MAb binding correlated poorly with anti-MSP-1_{19} OD values (Fig. 1), with most of the association being

FIG. 1. Competition for binding to rMSP-1_{19} between mouse MAbs and IgG antibodies in human sera from The Gambia. Competition assays were performed at a serum dilution of 1:50. Anti-MSP-1_{19} ODs were determined at a serum dilution of 1:1,000. A total of 236 Gambian sera were tested. (a and b) Correlation between anti-MSP-1_{19} OD and percent competition with MAb 12.10 (a) and MAb 8A12 (b). (c) Five sera which gave a low anti-MSP-1_{19} OD at 1:1,000 but showed >50% competition with MAb 12.10 at 1:50 were serially titrated. The titrated sera are indicated in the inset (which is a detail from panel a). Serum 1220, which showed <50% competition with MAb 12.10 (and thus is not shown in the inset), was titrated as a negative control. O, serum 1254; ■, serum 1073; ●, serum 1052; ▲, serum 1176; x, serum 1252; □, 1220; ◇, positive control serum pool.

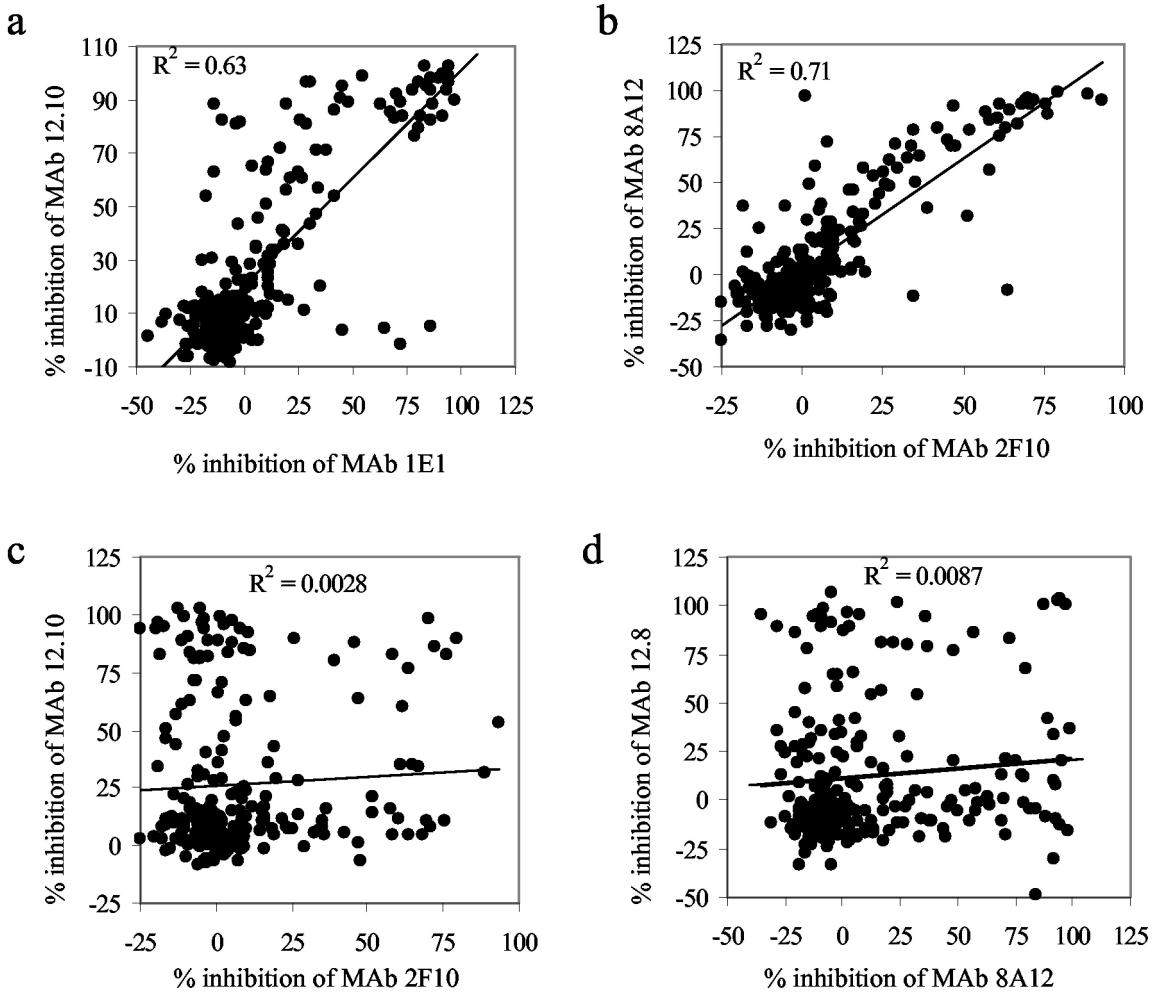


FIG. 2. Correlation for inhibition of binding of different MAbs to rMSP-1_{19} by human antibodies. Correlation of percent competition by sera from Gambian children for MAb 12.10 versus MAb 1E1 (a), MAb 8A12 versus MAb 2F10 (b), MAb 12.10 versus MAb 2F10 (c), and MAb 12.8 versus MAb 8A12 (d).

explained by sera that neither bound rMSP-1_{19} nor inhibited MAb binding. Some sera that showed strong direct binding to rMSP-1_{19} were unable to compete with any of the MAbs while, intriguingly, some sera that showed a very low level of binding to rMSP-1_{19} nevertheless were able to block MAb binding. Since direct binding to rMSP-1_{19} was determined at a serum concentration of 1:1,000 and competition assays were carried out with serum concentrations of 1:50 or 1:250, we suspected that these sera contained low concentrations of very-high-affinity antibodies to rMSP-1_{19}.

To examine associations among antibody concentration, affinity, and ability to compete with anti-MSP-1_{19} MAbs, we titrated a number of individual sera which were able to compete with MAb 12.10 despite weak or modest direct binding to rMSP-1_{19} (Fig. 1c). All of the sera that were able to block the binding of MAb 12.10 by >50% showed significantly greater binding to rMSP-1_{19} at a 1:50 or a 1:250 dilution than at a 1:1,000 dilution, indicating that they contained low concentrations of anti-MSP-1_{19} antibodies. That these sera were able to compete effectively with MAb 12.10 suggests that the antibodies have high affinity and restricted specificity. The importance of antibody affinity was confirmed by the observation that many more sera with low concentrations of anti-MSP-1_{19} antibodies were able to compete with MAb 8A12 (Fig. 1b) than with MAb 12.10 (Fig. 1a), as MAb 8A12 has an approximately 10-fold-lower affinity for rMSP-1_{19} than does MAb 12.10 (P. H. Corran, unpublished data).

The ability of individual sera to compete with different MAbs was highly correlated for certain pairs of MAbs, e.g., 1E1 with 12.10 (Fig. 2a) and 8A12 with 2F10 (Fig. 2b), indicating that the epitopes of these MAb pairs may overlap. For other pairings, e.g., 12.10 with 2F10 (Fig. 2c) or 12.8 with 8A12 (Fig. 2d), competition was very poorly correlated, indicating minimal overlap of the epitopes of these MAbs.

The proportion of sera that competed with each MAb did not change with age, except that the prevalence of competition with MAb 1E1 increased significantly with increasing age ($P = 0.018$) (Table 1). There was no significant association between MAb competition and sex or sickle cell hemoglobin genotype (data not shown). Serum antibodies from Fula (F) children were significantly more likely to compete with MAb 1E1 than were serum antibodies from Mandinka (M) and

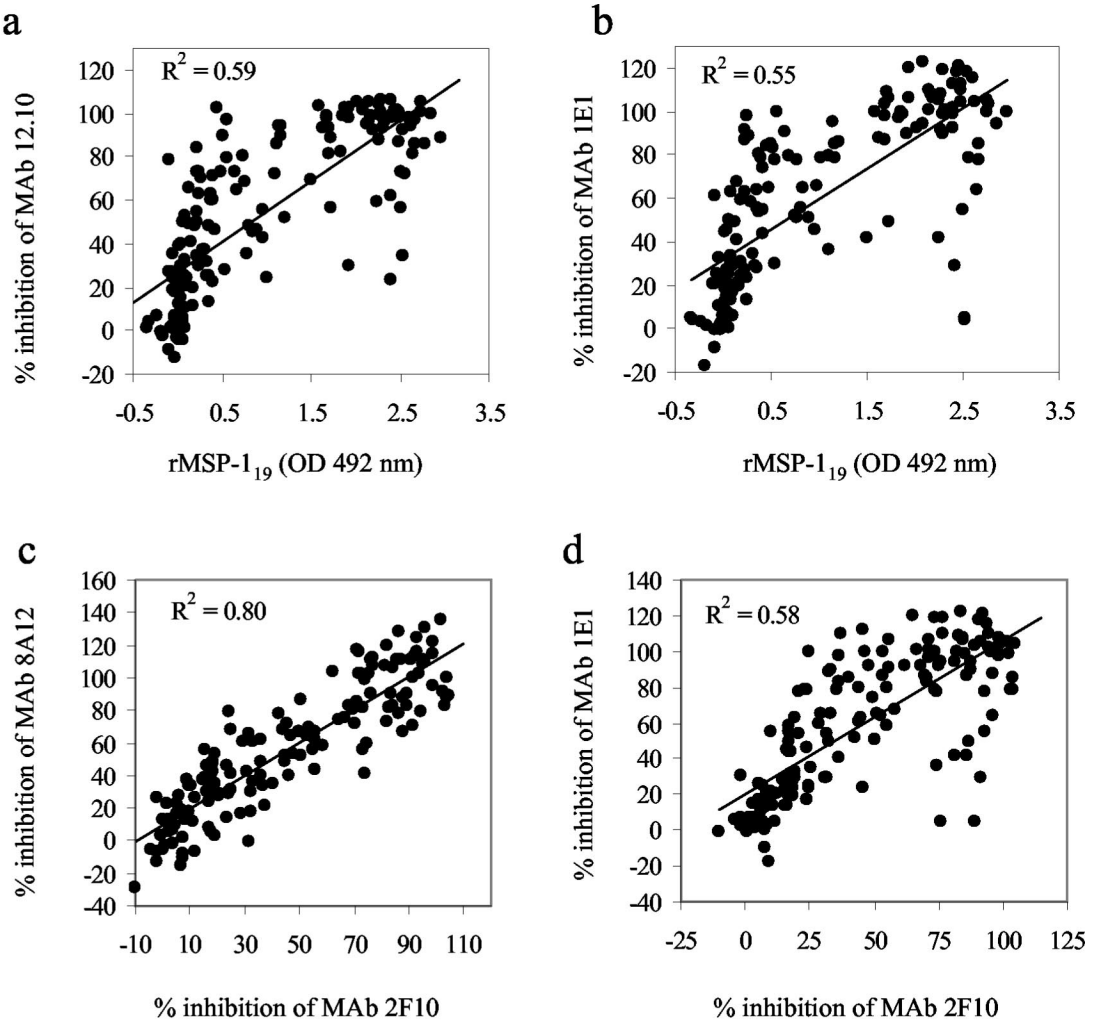


FIG. 3. Competition between mouse MAbs and IgG antibodies in human sera from Uganda for binding to rMSP-1_{19}. Competition assays were performed at a serum dilution of 1:50. Anti-MSP-1_{19} ODs were determined at a serum dilution of 1:1,000. A total of 156 Ugandan sera were tested. (a and b) Correlation between anti-MSP-1_{19} OD and percent competition for MAb 12.10 (a) and MAb 1E1 (b). Correlation of percent competition for MAb 8A12 versus MAb 2F10 (c) and MAb 1E1 versus MAb 2F10 (d).

Wolof (W) children (F, 24%; M, 6%; W, 10%; $\chi^2 = 11.7$; $P < 0.003$) and MAb 12.10 (F, 38%; M, 14%; W, 20%; $\chi^2 = 12.3$; $P = 0.002$).

The ability of Ugandan sera to compete with any of the MAbs was highly correlated with the anti-MSP-1_{19} titer ($R^2 = 0.47$ to 0.69; $P < 0.001$) (examples for two of the six MAbs tested are shown in Fig. 3a and b), and competition was highly correlated for all MAb pairs ($R^2 = 0.51$ to 0.80) (examples for two of the six MAbs tested are shown in Fig. 3c and d). There was no significant change in the prevalence of competing antibodies with age (Table 2).

Relationship between MAb competition and malaria morbidity. Gambian children whose antibodies competed with blocking MAb 1E1 were significantly less likely to become infected with malaria than were those whose antibodies did not compete ($P = 0.04$) (Table 1), and this difference remained significant after adjustment for the effects of age ($P = 0.03$). These results suggest that children who showed no evidence of infection throughout the high-transmission season have immunity to blood-stage infection which may be mediated by a subpopulation of anti-MSP-1_{19} antibodies with binding sites that overlap with binding site of MAb 1E1; these antibodies presumably interfere with the binding of blocking antibodies but do not themselves mediate blocking activity.

For the Ugandan cohort, MAb competition was compared with peak parasite density during follow-up by stratifying parasite density into three categories (<1,000 asexual blood-stage parasites/μl, 1,000 to 4,999 parasites/μl, and ≥5,000 parasites/μl) and comparing the proportions of sera that competed in each group (Table 2). There were statistically significant inverse associations between peak parasite density and competition (at a 1:250 serum dilution but not at 1:50) with MAbs 8A12, 1E1, 12.10, and 2F10 (P values for all comparisons, ≤0.023). These associations remained significant after adjustment for age, sex, village of residence, and bed net use. Thus, the ability of antibodies to protect against high-density parasitemia seems to be dependent on their concentration or affinity. Antibodies that compete with any of the four MAbs may

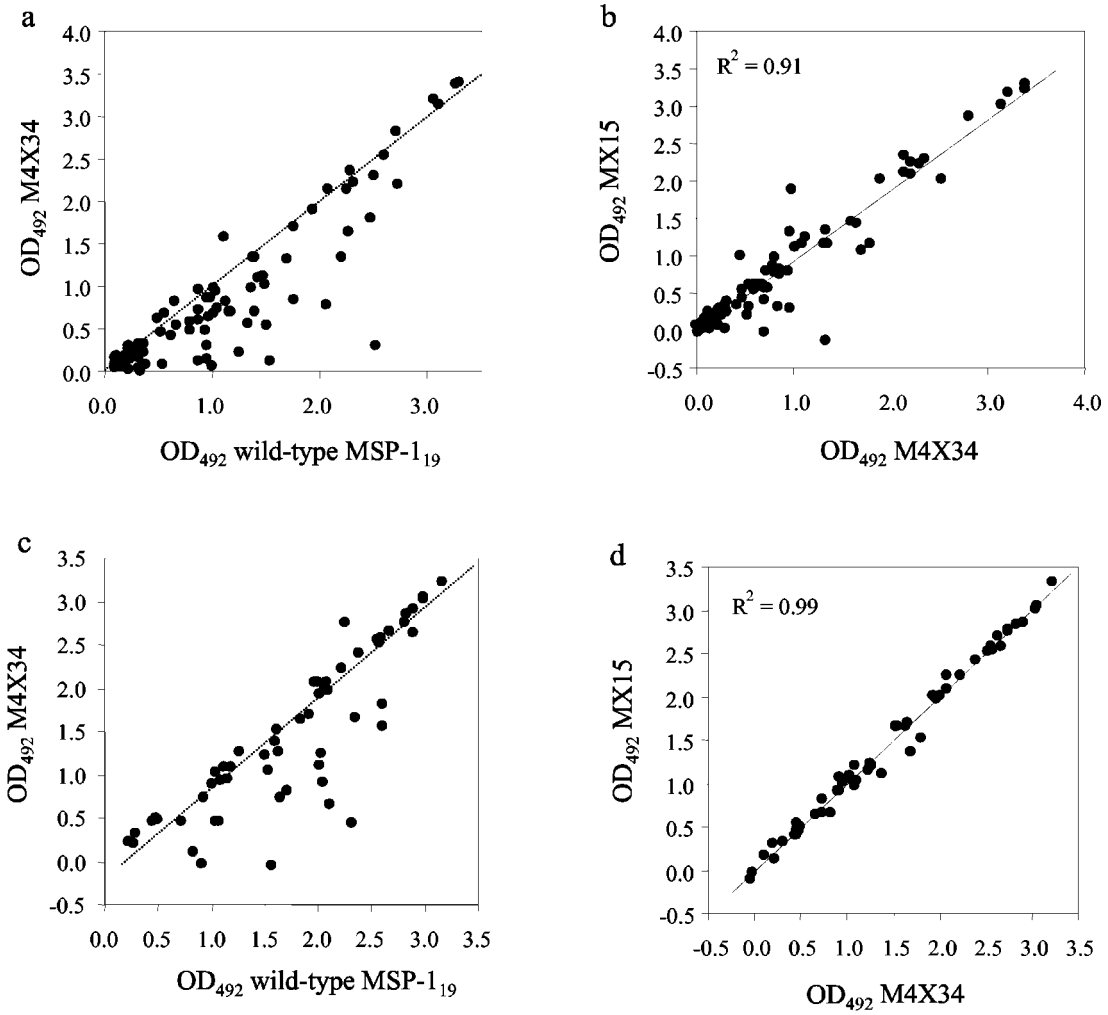


FIG. 4. Correlation of binding of human serum IgG to wild-type rMSP-1_{19} and rMSP-1_{19} mutants. IgG ODs for sera at a 1:1,000 dilution were determined with an ELISA. (a) Wild-type rMSP-1_{19} versus M4X34 for 55 Gambian sera. (b) M4X15 versus M4X34 for 55 Gambian sera. (c) Wild-type rMSP-1_{19} versus M4X34 for 56 Ugandan sera. (d) M4X15 versus M4X34 for 56 Ugandan sera. The diagonal lines indicate the distributions of values expected if antibodies bind equally to the proteins. Binding to different mutant proteins (b and d) was highly correlated, but a poor correlation was observed for binding to wild-type and mutant proteins (a and c) for some sera.

be able to protect against high-density parasitemia; however, as competition with each of the MAbs is highly correlated within an individual, it is possible that only some of the antibody specificities are directly involved in protection.

Human antibody recognition of rMSP-1_{19} mutants. As an alternative strategy for defining the fine specificity of human antibodies to rMSP-1_{19}, we tested the binding of serum IgG from Gambian and Ugandan children to three rMSP-1_{19} mutants that no longer bind the blocking MAbs 1E1 and 7.5 but that still bind the invasion-inhibiting MAbs 12.8 and 12.10 (26). By analogy, sera that recognize wild-type rMSP-1_{19} but that do not recognize the mutant proteins have antibodies whose specificity overlaps that of the blocking MAbs, while sera that continue to recognize the mutant proteins contain antibodies whose specificity overlaps that of the inhibitory MAbs. We hypothesized that the binding of serum antibodies to rMSP-1_{19} mutants might be associated with resistance to high-density parasitemia or clinical malaria.

Sera with antibodies to wild-type rMSP-1_{19} (Gambian sera, $n = 78$; Ugandan sera, $n = 56$) were tested for binding to the mutant proteins, and the ODs for the mutant proteins were plotted against the ODs for wild-type rMSP-1_{19} (Fig. 4a and c). Some sera bound equally well to mutant and wild-type antigens (putative inhibitory profile); others bound less well to the mutant antigens (putative blocking profile). Responses to the mutant proteins were highly correlated (Fig. 4b and d), indicating that the three mutant proteins have similar epitopes. There was no association between age and antibody binding to the mutant proteins (data not shown), confirming that age is not a major factor influencing the epitope specificity of antibodies to rMSP-1_{19}.

Binding to rMSP-1_{19} mutants identifies protective antibodies. As responses to the three mutant proteins were highly correlated, data for just one protein (M3) are shown (Table 3). All seropositive sera from The Gambia were tested for binding to M3; for the Ugandan subjects, only sera that showed a high

TABLE 3. Binding of serum antibodies to wild-type and mutant (M3) rMSP-1_{19} and association with clinical outcome

Parameter	*n*	No. (%) of antibodies with the following characteristics:			
		Wild-type MSP-1_{19}[a] (group 1)	OD ratio of[b]:		
			<25% (group 2)[c]	25–75% (group 3)	>75% (group 4)
Clinical category (The Gambia)					
No infection	51	35 (25)	1 (14)	3 (18)	12 (29)
Asymptomatic infection	53	36 (26)	1 (14)	6 (35)	10 (24)
Clinical malaria	75	49 (35)	3 (43)	6 (35)	17 (41)
Fever plus low level of parasitemia	25	19 (14)	2 (29)	2 (12)	2 (5)
Total	204	139 (100)	7 (100)	17 (100)	41 (100)
Maximum parasitemia (parasites/μl) (Uganda)					
<1,000	43	17 (39)	1 (2)	9 (21)	16 (37)
1,000–5,000	54	33 (62)	3 (6)	4 (7)	14 (26)
>5,000	21	12 (57)	0	3 (14)	6 (29)
Total	118	62	4	16	36

[a] Antibodies that were seronegative for rMSP-1_{19} (OD, ≤0.263).
[b] OD for binding to M3 protein as percentage of OD for binding to wild-type rMSP-1_{19} for the subgroup of antibodies that had high titers to rMSP-1_{19} (OD, >1.3).
[c] χ^2 (*P*) values were 0.03 (0.86) for differences between groups 1 and 2 versus groups 3 and 4 (The Gambia) for comparison of no infection with any infection (asymptomatic, clinical malaria, or fever with low parasitemia) and 5.44 (0.02) for differences between groups 1 and 2 versus groups 3 and 4 (Uganda) for maximum parasitemia of <1,000/μl versus ≥1,000/μl.

level of binding (OD, >1.3) to wild-type rMSP-1_{19} were tested for binding to M3. Sera were divided into those that were seronegative for anti-MSP-1_{19} antibodies (group 1), those that were seropositive for wild-type rMSP-1_{19} but bound to M3 with an OD <25% that of the wild type (group 2; putative blocking profile), those that bound to M3 with an OD of between 25 and 75% that of the wild type (group 3; mixed profile of both blocking and inhibitory antibodies), and those that bound to M3 with an OD >75% that of the wild type (group 4; inhibitory profile). We hypothesized that children in groups 1 and 2 would not be protected from infection or high-density parasitemia and that children in group 4 would be protected.

The number of seropositive Gambian children (groups 2, 3, and 4) was too small for any significant difference in clinical outcome to be detected between the groups. Among Ugandan children, the number of children in each of the four groups also was low, but when children in groups 3 and 4 were combined, they were found to be significantly less likely to experience high-level parasitemia during follow-up than were those in groups 1 and 2 combined ($\chi^2 = 5.44$; $P = 0.02$).

DISCUSSION

Epidemiological studies have yielded very inconsistent results with regard to the association between anti-MSP-1_{19} antibodies and clinical immunity to malaria (2, 5, 9, 13, 16), and a recent study has shown no correlation between titers of anti-MSP-1_{19} antibodies and inhibition of MSP-1 processing (22), which is known to be one of the main effector mechanisms mediated by these antibodies (1). Here we have confirmed the lack of association between total anti-MSP-1_{19} antibodies and resistance to clinical malaria in The Gambia (9) and have shown no evidence of any protective effect of these antibodies in Uganda. We hypothesized that the fine specificity of the antibody response is a crucial determinant of antibody efficacy, and we examined associations between antibody specificity and resistance to malaria infection in two unrelated populations by using two methods.

The competition ELISA was based on the premise that serum antibodies that share overlapping epitope specificities with MAbs will bind sufficiently close to the MAbs to sterically interfere with MAb binding. The degree of competition will be influenced by the relative affinities of the serum antibodies and the MAbs, the degree of overlap of the epitopes, and the concentration of antibodies in the serum. Importantly, antibodies that compete may (or may not) share the same functions as the MAb; if the overlap is complete, then shared functions are to be expected, but if the overlap is only partial, then the effector functions may be very different.

The second assay, the binding of serum antibodies to rMSP-1_{19} mutants, in which the binding site for blocking MAbs had been disrupted, assumed that sera which bound to wild-type rMSP-1_{19} but not to rMSP-1_{19} mutants contained predominantly blocking antibodies. Again, however, the extent to which antibodies with similar but not identical binding characteristics actually share the same functions is not known.

In both Gambian and Ugandan sera, there was marked heterogeneity in the ability of anti-MSP-1_{19} antibodies to compete with the different MAbs and to bind to the mutant antigens, confirming our hypothesis of variations between individuals in the fine specificity of their anti-MSP-1_{19} antibodies. The higher prevalence of competing antibodies in Gambian children of Fula origin than in other ethnic groups and the bimodal distribution of anti-MSP-1_{19} antibodies in Ugandan children suggest that anti-MSP-1_{19} antibody responses may be genetically regulated. This suggestion was made previously for anti-MSP-1_{19} antibodies (9). Others have shown that Fula populations make stronger antibody responses to malaria while being less frequently parasitized and less susceptible to clinical malaria (20, 21).

Importantly, in both cohorts, significant associations were

observed between antibody specificity and protection from infection (in The Gambia) or from high-density parasitemia (in Uganda), and several of the associations were seen in both cohorts. However, given the multiple comparisons made in this study and the fact that some findings could not be replicated in both study populations, it is possible that some of the significant associations that we have observed may have arisen by chance; our findings do need to be corroborated by other independent studies. Replication of findings from immunoepidemiological studies at more than one site is essential, as it is quite possible for spurious associations to occur due to the effect of unknown, population-specific confounding factors. However, it is also possible that real associations may be missed in some studies due to a lack of statistical power. Furthermore, as clinical immunity to malaria is undoubtedly multifactorial—with multiple potential effector mechanisms and target antigens—the contribution of any one effector mechanism to the clinical outcome may not always be evident. Any of these factors may explain why some of the findings observed in the Ugandan study could not be replicated in the Gambian study; however, the lower MSP-1_{19} seroprevalence in The Gambia and, especially, the small numbers of sera with particular competition or mutant-binding profiles do markedly reduce the power of this study compared to the Ugandan study.

In children in The Gambia, resistance to infection was associated with competition with blocking MAb 1E1 (and, to a lesser extent, 12.10). The simplest interpretation of this finding is that these children possess antibodies that recognize an epitope that overlaps those recognized by 1E1 and 12.10 and inhibit merozoite invasion. This interpretation is supported by the Ugandan study, where children whose sera competed with 1E1 or 12.10 at a titer of at least 1:250 were significantly less likely to develop high-density parasitemia than were children whose sera did not compete. The association between resistance and the presence of antibodies with similarities to 1E1 (which is not protective but in fact blocks the binding of protective antibodies) is somewhat counterintuitive; such antibodies might be expected to correlate with susceptibility to infection rather than protection. We suspect that human antibodies that compete with 1E1 are not themselves blocking antibodies but may interfere with the binding of blocking antibodies and thus confer a degree of protection. In vitro growth inhibition and processing assays are under way in our laboratory to determine whether this is the case.

We used rMSP-1_{19} mutants to produce an independent map of the anti-MSP-1_{19} antibody response. The combination of mutations that abolished the binding of blocking MAbs significantly reduced the binding of some sera, and the children with these sera were at significantly greater risk of developing high-density parasitemia. These findings indicate that the results of the competition assays and the mutant-binding studies actually provide slightly different data about human antibodies and suggest that a comparison of binding to wild-type versus mutant proteins allows the identification of sera that contain blocking antibodies. Furthermore, these findings indicate that blocking antibodies are induced by natural infection and that this process might be a successful immune evasion strategy for malaria parasites.

Interestingly, in Uganda, competition with MAb 12.10 was highly correlated with resistance to high-density parasitemia, but there was no such association with MAb 12.8. These two MAbs have very similar profiles in bioassays and block each other's binding in competition assays (10), indicating that their epitopes overlap. However, these two MAbs have subtle differences in binding characteristics, as shown by a two-site radioimmunoassay (28) or Western blot analysis of binding to MSP-1_{19} mutants (26). The data presented here suggest that these minor differences in binding specificity also may be functionally relevant; if this is the case, then the protective effect of anti-MSP-1_{19} antibodies may be exquisitely sensitive to the fine specificity of the antibodies, a situation which may pose real problems for the development of protective vaccines.

In summary, we have shown that there are extensive variations between individuals in the fine specificity of the naturally acquired antibody response to MSP-1_{19}, and we have demonstrated significant associations between antibody specificity (and titer) and protection against malaria infection and high-density parasitemia. Thus, the fine specificity of the antibody response is an important criterion for the functional efficacy of anti-MSP1_{19} antibodies. This study provides a strong rationale for using specifically mutated proteins—which no longer induce blocking antibodies—as synthetic immunogens to induce an antibody response that is significantly more protective than that induced by exposure to wild-type parasites. Finally, this study represents a significant step forward in the search for in vitro correlates of protective immunity that are amenable to high-throughput analysis and rigorous quality control, features which are essential for the efficient evaluation of potential vaccine antigens.

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