Acquired Antibodies to Merozoite Antigens in Children from Uganda with Uncomplicated or Severe Plasmodium falciparum Malaria

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Malaria can present itself as an uncomplicated or severe disease. We have here studied the quantity and quality of antibody responses against merozoite antigens, as well as multiplicity of infection (MOI), in children from Uganda. We found higher levels of IgG antibodies toward erythrocyte-binding antigen EBA181, MSP2 of Plasmodium falciparum 3D7 and FC27 (MSP2-3D7/FC27), and apical membrane antigen 1 (AMA1) in patients with uncomplicated malaria by enzyme-linked immunosorbent assay (ELISA) but no differences against EBA140, EBA175, MSP1, and reticulocyte-binding protein homologues Rh2 and Rh4 or for IgM against MSP2-3D7/FC27. Patients with uncomplicated malaria were also shown to have higher antibody affinities for AMA1 by surface plasmon resonance (SPR). Decreased invasion of two clinical P. falciparum isolates in the presence of patient plasma correlated with lower initial parasitemia in the patients, in contrast to comparisons of parasitemia to ELISA values or antibody affinities, which did not show any correlations. Analysis of the heterogeneity of the infections revealed a higher MOI in patients with uncomplicated disease, with the P. falciparum K1 MSP1 (MSP1-K1) and MSP2-3D7 being the most discriminative allelic markers. Higher MOIs also correlated positively with higher antibody levels in several of the ELISAs. In conclusion, certain antibody responses and MOIs were associated with differences between uncomplicated and severe malaria. When different assays were combined, some antibodies, like those against AMA1, seemed particularly discriminative. However, only decreased invasion correlated with initial parasitemia in the patient, signaling the importance of functional assays in understanding development of immunity against malaria and in evaluating vaccine candidates.

Malaria is a parasitic disease caused by the intracellular protozoan Plasmodium. Most of the deaths are caused by Plasmodium falciparum, and the majority of cases occur in children and pregnant women in sub-Saharan Africa (1, 2). P. falciparum invasion of erythrocytes involves different invasion pathways, with multiple interactions between merozoite antigens and erythrocyte receptors (3). Two main protein families involved in invasion are erythrocyte binding-like (EBL) proteins and P. falciparum reticulocyte-binding protein homologue (RBP/PfRh) proteins. The erythrocyte-binding antigens (EBAs) are part of the EBL family and include EBA140, EBA175, and EBA181, while PfRh1, PfRh2, PfRh4, and PfRh5 are among the PfRh proteins (4–6). Changes in invasion pathways have been shown to influence the susceptibility of P. falciparum to human invasion-inhibitory antibodies (7). Other proteins that are central in the invasion process include merozoite surface proteins (MSPs), such as MSP1 (8) and MSP2 (9). The merozoite proteins are highly polymorphic, and MSP1 can be divided into three allelic types (K1, MAD20, and RO33 [MSP1-K1, -MAD20, and -RO33, respectively]), and MSP2 can be divided into two allelic types (3D7 and FC27 [MSP2-3D7 and -FC27, respectively]) (10, 11). Apical membrane antigen 1 (AMA1) is a protein that has been described to be essential for invasion; in comparison to many other merozoite antigens, AMA1 is found in all Plasmodium species, and its sequence is relatively conserved between different parasite lines (12, 13) even though several polymorphisms have been described in the ectodomain (14, 15).

Individuals living in areas of malaria endemicity develop immunity but only slowly and after repeated exposure. Passive transfer of antibodies from immune donors to individuals with P. falciparum infection has been shown to reduce parasitemia and clinical symptoms (16–18). Immunity against severe malaria usually develops before total protection against disease is established (19), indicating either that different P. falciparum antigens are important in protection from severe compared to uncomplicated malaria or that the quality of the antibodies in the two groups is different. Antibodies against several merozoite antigens have been found to be associated with protective immunity in prospective longitudinal studies (20–30). However, very few studies have examined the functional properties of acquired antibodies (31) or examined the role of antibodies to merozoite antigens in immunity to severe malaria in young children. Invasion inhibition assays (IIAs) and growth inhibition assays (GIAs) can be applied to study the function of antibodies in vitro. Studies have described the invasion/growth-inhibitory activities in sera from individuals living in areas of malaria endemicity (7, 17, 32–37). These naturally acquired inhibitory antibodies (causing reduced invasion of parasite isolates) are present in many clinically immune individuals but more seldom in susceptible individuals, and levels are higher in areas with higher levels of malaria transmission (36). Some studies have shown a protective effect of inhibitory antibod-
ies (33, 38, 39); however, the association between the inhibitory activity of antibodies and protection in malaria remains unclear (35, 36, 40).

The lack of in vitro functional assays that correlate with protective immunity in vivo has hampered the development of effective blood stage vaccines (1). There have been inconsistencies in the correlations of antibody responses to recombinant antigens and protection from malaria using enzyme-linked immunosorbent assays (ELISAs) (36). Trials that aim to improve the value of ELISAs have included the use of ammonium thiocyanate (NH₄SCN) ELISAs to estimate avidity of antibodies (41), but the introduction of surface plasmon resonance (SPR) (42) has opened new opportunities to measure the affinity of antibodies under flow, something that ought to be more similar to the physiological situation than static ELISAs. SPR is a method whereby association and dissociation between antibody and antigen can be studied in real time, and it has been essential in vaccine development studies for other pathogens, such as HIV (42). In malaria, SPR has mainly been used for studies of monoclonal antibodies (43, 44), but a recent study of naturally acquired polyclonal antibodies showed that individuals with high-affinity antibodies directed against MSP2-3D7 showed prolonged time to developing clinical malaria (45), indicating that the presence of high-affinity antibodies may be important in protection against malaria. The method used is probably of importance since another study using guanidine thiocyanate for evaluation of the strength of antibody binding did not show any correlation to clinical malaria (46).

Whether a patient will develop uncomplicated or severe malaria is influenced not only by the immune responses of the host but also by genetic differences in the parasites, which might in turn be dependent on the transmission level in the area since high transmission levels will cause more frequent recombination events in the mosquito. Patients often harbor many variants of parasites at the same time (47–52), and characterization of different clones of *P. falciparum* could be a useful tool to understand the molecular epidemiology of malaria. However, results reported in the literature have been conflicting as to whether multiclonal infections are associated with the outcome of the infection (53–60).

A way of determining the importance of the heterogeneity of infections is to measure the multiplicity of infection (MOI), which is an estimation of how many genetically different parasites the patient is harboring, and investigate potential associations between MOIs and the outcomes of an infection.

In this study, we have examined antibody responses to merozoite antigens and MOIs among children with uncomplicated versus severe *P. falciparum* malaria. We have studied quantitative and qualitative differences in antibody responses against merozoite antigens, including ELISA, IIA, NH₄SCN ELISA, and SPR, to identify antibody responses that may play a role in protective immunity and evaluate which method(s) is predictive of immune differences between severe and uncomplicated malaria. This will enable us to better understand how immunity in malaria is developed and which antigens should be prioritized in future vaccine trials.

**MATERIALS AND METHODS**

**Study site and population.** A total of 85 children (6 months to 3 years of age) with active *P. falciparum* infection were included in this study, as described previously (61). They were recruited from the district hospital in Apac, northern Uganda, in an area of holoendemicity, and the patients were grouped according to WHO guidelines (62). This area has been shown before to have a very high entomological inoculation rate (EIR) of >1,500 (63, 64). Forty-six patients were classified as having severe malaria, and 39 patients were defined as having uncomplicated malaria. Written informed consent to participate in the study was received from all guardians, and ethical permissions were granted for the study from Makerere University Faculty of Medicine Research and Ethics Committee in Uganda (number MV 717) and the Stockholm Ethical Review Board (permission number 03-095).

**Invasion inhibition assay.** The method to study invasion-inhibitory antibodies has been described previously (7, 37). Two Ugandan *P. falciparum* isolates (UAM37, from a patient with uncomplicated malaria, and UAS31, from a patient with severe malaria) were chosen as representative of isolates in this study group (61). Isolates were cultured in vitro in AB⁺ nonimmune Swedish serum and gassed with 90% NO₂, 5% O₂, and 5% CO₂ and placed in a shaker incubator. In brief, parasites were synchronized (5% [vol/wt] sorbitol) before start of the assay, and at the day of the assay the majority of the parasites were at late-pigmented trophozoite stage. Parasite suspensions (50 μl) were cultured for one cycle in 96-well plates. Five microliters of dialedyzed test plasma was added to each well, and all samples were run in duplicate. Plates were incubated in a sealed, humidified, gassed box and put in an incubator for 48 h at 37°C. Parasitemia was estimated using hydroethidine (10 μg/ml; Sigma-Aldrich) in a flow cytometer (FACScan; BD) after approximately 48 h (determined by the parasite stage). Parasite invasion for each sample was measured in comparison to controls (invasion in the presence of dialedyzed Swedish plasma).

If there was no invasion-inhibitory activity in the added plasma samples, the invasion of the parasites was 100%.

**Antibodies in Uncomplicated and Severe Malaria**

**Recombinant proteins.** All recombinant proteins were expressed in *Escherichia coli*, including following: the whole ectodomain of the *P. falci-
parum* D10 allelic form of AMA1 (AMA1-D10) (12); regions III to V of EBA140 (3D7 allelic type; amino acids [aa] 761 to 1298), and EBA181 (3D7; aa 769 to 1365) (29); and Rh2a9 (3D7; aa 2027 to 2533) (65) and Rh4a3 (aa 1160 to 1370) (7, 66). The MSP2 proteins corresponding to the FC27 and 3D7 gene sequences (10) were expressed as described previously. MSP1-19 from the 3D7 sequence was amplified from LIEKGF-DGIFCS with flanking SacII and PstI sites and ligated in pASK45+ from IBA, Germany. MSP1-19 was purified and refolded as described previously (67). The recombinant proteins were glutathione S-transferase (GST) tagged.

**Antibodies against recombinant proteins determined by ELISA.** ELISAs for MSP2-FC27, MSP2-3D7, and AMA1-D10, were performed as described previously (45). For MSP1, the ELAs, and Rh proteins, the method described by Persson et al. was used (7). Optical density (OD) was measured at 405 nm (OD₄₀₅). Test samples at a dilution 1:100 were run in duplicate together with positive and negative controls to allow for standardization (a pool of exposed adult individuals from Uganda and 5 to 7 nonimmune Swedish donors, respectively). Antibody reactivity was considered positive when the OD was greater than the mean plus 3 standard deviations (SDs) of the value for the nonimmune Swedish donors.

**Ammonium thiocyanate (NH₄SCN) ELISA.** To estimate the strength of the binding, increasing concentrations of NH₄SCN were added to ELISAs for the MSP2-3D7 and MSP2-FC27 proteins as described previously (45). The affinity index was calculated from the absorbance readings in the presence of increasing concentrations of NH₄SCN, which were converted to percentages of total bound antibody (in the absence of NH₄SCN). The index was calculated from the molar concentration of NH₄SCN required to reduce the initial absorbance by 50% (68).

**Surface plasmon resonance.** To estimate the affinity of antibodies in plasma binding to different antigens, the recombinant MSP2-3D7, MSP2-FC27, and AMA1-D10 were bound to CM5 chips in surface plasmon resonance assays (Biacore 3000, Uppsala, Sweden) as described previously (45). One lane was used as a control lane. Around 1,000 response units (RU) of each protein was bound to the chip. Plasma samples in at least two different dilutions (used as internal controls since the off-rate kd dissoci-
TABLE 1 Classification and parasitemia of all patients included in the study

| Patient group and identification no. | Sexa | Age (mo.) | Initial parasitemia (%) | Disease state b |
|-------------------------------------|------|-----------|-------------------------|----------------|
| Uncomplicated malaria (n = 39)      |      |           |                         |                |
| CO 05 F                            | 12   | 1         | Mild malaria            |                |
| CO 06 M                            | 36   | 2         | Mild malaria            |                |
| CO 09 F                            | 4    | 2.4       | Mild malaria            |                |
| CO 11 M                            | 14   | 2.8       | Mild malaria            |                |
| CO 13 F                            | 5    | 1         | Mild malaria            |                |
| CO 14 F                            | 35   | 1.7       | Mild malaria            |                |
| CO 15 M                            | 24   | 1.4       | Mild malaria            |                |
| CO 16 F                            | 9    | 1.6       | Mild malaria            |                |
| CO 17 M                            | 9    | 1         | Mild malaria            |                |
| CO 18 M                            | 27   | 2         | Mild malaria            |                |
| CO 19 F                            | 24   | 1         | Mild malaria            |                |
| CO 20 M                            | 27   | 1         | Mild malaria            |                |
| CO 21 M                            | 6    | 3.2       | Mild malaria            |                |
| CO 22 M                            | 11   | 4         | Mild malaria            |                |
| CO 24 F                            | 5    | 6.7       | Mild malaria            |                |
| CO 25 F                            | 11   | 4.2       | Mild malaria            |                |
| CO 27 F                            | 15   | 1.5       | Mild malaria            |                |
| CO 28 M                            | 24   | 2.3       | Mild malaria            |                |
| CO 29 F                            | 12   | 2.5       | Mild malaria            |                |
| CO 31 F                            | 9    | 1.9       | Mild malaria            |                |
| CO 32 F                            | 20   | 1         | Mild malaria            |                |
| CO 33 F                            | 7    | 3.8       | Mild malaria            |                |
| CO 34 M                            | 7    | 2         | Mild malaria            |                |
| CO 35 F                            | 10   | 1.2       | Mild malaria            |                |
| CO 36 M                            | 12   | 4.8       | Mild malaria            |                |
| CO 38 M                            | 8    | 1.8       | Mild malaria            |                |
| CO 39 F                            | 7    | 1.5       | Mild malaria            |                |
| CO 40 F                            | 24   | 5.1       | Mild malaria            |                |
| CO 41 M                            | 18   | 1.1       | Mild malaria            |                |
| CO 42 M                            | 9    | 5.9       | Mild malaria            |                |
| CO 43 M                            | 8    | 4.1       | Mild malaria            |                |
| CO 44 M                            | 6    | 2.3       | Mild malaria            |                |
| CO 45 F                            | 29   | 1.8       | Mild malaria            |                |
| CO 46 F                            | 30   | 3         | Mild malaria            |                |
| CO 47 F                            | 12   | 1.3       | Mild malaria            |                |
| CO 48 F                            | 20   | 3.6       | Mild malaria            |                |
| CO 49 F                            | 6    | 1.6       | Mild malaria            |                |
| CO 50 F                            | 6    | 2         | Mild malaria            |                |
| Mean for group                     |      | 14.8      | 2.5                     |                |
| Severe malaria (n = 46)            |      |           |                         |                |
| SE 01 M                            | 8    | 6         | Severe malaria NUD      |                |
| SE 02 F                            | 8    | 6         | Severe malaria NUD      |                |
| SE 04 M                            | 24   | 3         | Respiratory distress    |                |
| SE 05 F                            | 24   | 3         | Severe malaria NUD      |                |
| SE 07 F                            | 11   | 9         | Respiratory distress    |                |
| SE 08 F                            | 16   | 14.8      | Respiratory distress    |                |
| SE 09 M                            | 17   | 15        | Respiratory distress    |                |
| SE 11 F                            | 4    | 46        | Severe malaria NUD      |                |
| SE 12 F                            | 12   | 1         | Respiratory distress    |                |
| SE 13 M                            | 14   | 5         | Respiratory distress    |                |
| SE 14 F                            | 14   | 10        | Severe malaria NUD      |                |
| SE 15 M                            | 12   | 8         | Respiratory distress    |                |
| SE 16 F                            | 6    | 5         | Severe malaria NUD      |                |
| SE 17 F                            | 12   | 9         | Circulatory collapse    |                |
| SE 18 F                            | 5    | 1.4       | Respiratory distress    |                |
| SE 19 F                            | 14   | 1.1       | Respiratory distress    |                |
| SE 20 M                            | 6    | 1.9       | Severe malaria NUD      |                |
| SE 21 M                            | 8    | 9         | Respiratory distress    |                |
| SE 22 M                            | 17   | 21.8      | Respiratory distress    |                |
| SE 23 M                            | 20   | 4         | Cerebral malaria        |                |
| SE 24 F                            | 15   | 5         | Respiratory distress    |                |
| SE 25 M                            | 8    | 5.2       | Respiratory distress    |                |
| SE 26 F                            | 6    | 3.1       | Respiratory distress    |                |
| SE 27 M                            | 32   | 2         | Respiratory distress    |                |
| SE 28 F                            | 5    | 6.1       | Respiratory distress    |                |
| SE 29 F                            | 12   | 3.9       | Respiratory distress    |                |
| SE 30 M                            | 19   | 2         | Severe malaria NUD      |                |
| SE 31 M                            | 30   | 7.6       | Severe malaria NUD      |                |
| SE 32 M                            | 9    | 6.2       | Cerebral malaria        |                |
| SE 33 F                            | 14   | 2.8       | Respiratory distress    |                |
| SE 34 F                            | 8    | 10        | Respiratory distress    |                |
| SE 35 M                            | 30   | 8.3       | Respiratory distress    |                |
| SE 36 F                            | 5    | 9.3       | Respiratory distress    |                |
| SE 37 F                            | 14   | 5         | Respiratory distress    |                |
| SE 38 M                            | 30   | 9.5       | Severe malaria NUD      |                |

a M, male; F, female.  
b NUD, non ultra descriptus (without further description).

TABLE 1 (Continued)

| Patient group and identification no. | Sexa | Age (mo.) | Initial parasitemia (%) | Disease state b |
|-------------------------------------|------|-----------|-------------------------|----------------|
| SE 39 M                            | 6    | 9.3       | Respiratory distress    |                |
| SE 40 F                            | 9    | 5.5       | Respiratory distress    |                |
| SE 41 F                            | 11   | 7.9       | Circulatory collapse    |                |
| SE 42 F                            | 9    | 2.2       | Respiratory distress    |                |
| SE 43 F                            | 14   | 5.6       | Respiratory distress    |                |
| SE 44 M                            | 5    | 4.5       | Respiratory distress    |                |
| SE 45 F                            | 5    | 2.9       | Respiratory distress    |                |
| SE 46 M                            | 9    | 1.8       | Respiratory distress    |                |
| SE 47 F                            | 7    | 1.3       | Severe anemia           |                |
| SE 48 M                            | 15   | 7.3       | Respiratory distress    |                |
| SE 49 M                            | 9    | 5.7       | Respiratory distress    |                |
| Mean for group                     |      | 12.8      | 6.9                     |                |

a M, male; F, female.  

RESULTS

Patient information and initial parasitemia. In Table 1, information about the clinical status of the patients can be found. When the initial clinical parasitemia in the patients were compared in uncomplicated and severe malaria, a significant difference was found, with higher parasitemias in the severe malaria cases, as could be expected (Tables 1 and 2).

IgG and IgM antibody levels in uncomplicated and severe malaria estimated by ELISA. IgG levels against the recombinant merozoite antigens EBA140, EBA175, EBA181, PfRh2, PfRh4, MSP1, MSP2-3D7, MSP2-FC27, and AMA1 were assessed using ELISAs. For EBA181, MSP2-3D7, MSP2-FC27, and AMA1, the IgG levels were significantly higher in patients with uncomplicated malaria than in those with severe malaria (Table 2 and Fig. 1). As a positive control, a pool of immune donors was used and usually gave OD values of around 0.8 to 1.0. Swedish nonimmune plasma was used as a negative control, with OD values of <0.07.

An extended analysis was performed for the two MSP2 proteins for which we also performed IgM ELISAs, but no significant differences between uncomplicated and severe malaria were observed (Table 2).
There was a high prevalence of antibodies in general against all antigens tested (EBA140, 88%; EBA175, 94%; EBA181, 87%; Rh2, 92%; Rh4, 80%; MSP1, 98%; and for AMA1, MSP2-FC27, and MSP2-3D7, 100%). There were no major differences in prevalence rates between patients with uncomplicated malaria and those with severe malaria. The only difference that reached significance was for EBA140, with a slightly lower prevalence in mild cases (78%) than in severe cases (95%; $P = 0.02$).

Antibody affinity in uncomplicated and severe malaria. To further study the different properties of antibodies in uncomplicated and severe malaria, a subset of antigens was selected for studies of affinity of antibodies using surface plasmon resonance (SPR). Two allelic variants of the highly unstructured MSP2 protein, MSP2-FC27 and MSP2-3D7, were chosen together with AMA1. These antigens were selected since we had access to two alleles of the highly unstructured MSP2 proteins, and AMA1 was added because it is a globular protein with a stable tertiary structure. Affinity was estimated using the dissociation rate, $k_d$. Antibodies against AMA1 showed the highest affinity (i.e., lower $k_d$ values), while antibodies against MSP2-3D7 had lower affinity, and antibodies against MSP2-FC27 had the lowest affinity (Table 1).

| Parameter | Value for the indicated group (mean ± SD) | Uncomplicated malaria ($n = 39$) | Severe malaria ($n = 46$) | $P$ value$^e$ |
|-----------|------------------------------------------|---------------------------------|--------------------------|---------------|
| Age (mo.) |                                          | 14.8 (±9.0)                     | 12.8 (±7.9)              | 0.26          |
| IgG ELISA (OD$_{405}$ value)$^a$ | | | | |
| AMA1-D10 | 0.69 (±0.28)                             | 0.46 (±0.22)                    | 0.0002                   |
| MSP2-FC27 | 0.90 (±0.30)                             | 0.67 (±0.25)                    | 0.0005                   |
| EBA181 | 0.13 (±0.18)                             | 0.06 (±0.35)                    | 0.005                    |
| MSP2-3D7 | 0.8 (±0.32)                              | 0.67 (±0.21)                    | 0.03                     |
| EBA175 | 0.1 (±0.14)                              | 0.07 (±0.08)                    | 0.6                      |
| EBA140 | 0.06 (±0.09)                              | 0.06 (±0.05)                    | 0.2                      |
| MSP1 | 0.26 (±0.36)                              | 0.28 (±0.27)                    | 0.3                      |
| Rh2 | 0.18 (±0.24)                              | 0.18 (±0.23)                    | 1.0                      |
| Rh4 | 0.03 (±0.05)                              | 0.05 (±0.05)                    | 0.06                     |
| IgM ELISA (OD$_{405}$ value)$^a$ | | | | |
| MSP2-FC27 | 0.22 (±0.14)                             | 0.19 (±0.09)                    | 0.4                      |
| MSP2-3D7 | 0.21 (±0.12)                             | 0.19 (±0.12)                    | 0.09                     |
| NH$_4$SCN ELISA (affinity index)$^a$ | | | | |
| MSP2-FC27 | 0.9 (±0.68)                              | 0.88 (±0.54)                    | 0.8                      |
| MSP2-3D7 | 0.98 (±0.7)                              | 0.61 (±0.26)                    | 0.09                     |
| SPR ($k_d$ [s$^{-1}$])$^b$ | | | | |
| AMA1 | 0.00018 (±0.00011)                        | 0.00026 (±0.00022)              | 0.04                     |
| MSP2-FC27 | 0.00072 (±0.00022)                       | 0.00076 (±0.00023)              | 0.4                      |
| MSP2-3D7 | 0.00046 (±0.00022)                       | 0.00052 (±0.00023)              | 0.3                      |
| Invasion (%)$^c$ | | | | |
| UAS31 | 57.9 (±24.0)                             | 70.0 (±23.0)                    | 0.006                    |
| UAM37 | 72.0 (±32.0)                             | 72.0 (±29)                      | 0.7                      |
| Initial clinical parasitemia (%)$^d$ | | | | |
| 2.5 (±1.4) | 6.9 (±7.3)                                | 0.0003                     |

$^a$Total IgM/IgG levels to different recombinant antigens were estimated using ELISAs, and an extended analysis of MSP2 antigens was performed for estimation of affinity using NH$_4$SCN ELISAs.

$^b$ $k_d$ [s$^{-1}$] values were measured by SPR.

$^c$ Invasion was determined for two clinical isolates.

$^d$ Initial clinical parasitemia in the patients was determined by counting Giemsa slides.

$^e$ Differences between means were determined by a Mann-Whitney $t$ test. Significant $P$ values are in boldface.

FIG 1 Comparison of levels of IgG in plasma (ELISA) between patients with uncomplicated (UM) and severe (SE) malaria for a selection of recombinant antigens. Box plots show distribution of optical density (OD) values at 405 nm among uncomplicated and severe malaria cases. Horizontal bars in the middle of each box indicate the mean percentage of OD. The top and bottom of each box represent the upper and lower quartiles, respectively. The whiskers show the 5th and 95th percentiles. There were significant differences between uncomplicated and severe malaria for all antigens shown (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
assay setup was found within the first 8 h; hence, we chose to refer shown) and could see that a major part of the inhibition in our microscopy and flow cytometry at different time points (data not invasion inhibition assays with analysis of parasitemia using mi-

whether the decreased parasite invasion was due mainly to an and four from patients with severe malaria) was used to test against UAS31, but the difference was not significant (Table 2). A sion) when plasma from uncomplicated malaria patients was used (57.9%) compared to invasion in severe malaria cases (70% invasion). M required only 3D7 of MSP2 and RO33 of MSP1 (but not K1 or parasite UAM37 contained both the FC27 and 3D7 alleles of P. falciparum (s) characterized for uncomplicated and severe malaria separately or for all cases together. For each antigen, when uncomplicated and severe malaria cases were compared, only AMA1 showed a significant difference in \( k_d \) (s\(^{-1}\)) values (\( P = 0.02 \)).

2 and Fig. 2). For all three antigens, there was a trend of a higher affinity among the uncomplicated malaria samples, but values reached statistical significance only for AMA1. Previously, other studies have used NH\(_4\)SCN to estimate the affinity of antibodies, and we therefore wanted to include this method in our studies for comparison with the more precise SPR method. Increasing concentrations of NH\(_4\)SCN were added after incubation of MSP2-3D7 and MSP2-FC27 with total IgG. The affinity indexes were not significantly different in uncomplicated and severe malaria (Table 2). Invasion-inhibitory antibodies in uncomplicated and severe malaria. We used two clinical \( P. falciparum \) isolates (derived from patients in the study) to measure the invasion-inhibitory activity of antibodies in patient plasma. If there was no invasion-inhibitory activity in the added plasma samples, the invasion of the parasites was 100%. One isolate was from a patient with uncomplicated malaria (UAM37), and one was from a patient with severe malaria (UAS31). The plasma from the patients harboring the parasites UAM37 and UAS31 showed decreased parasite invasion in the presence of the plasma, with a range of 17 to 33% invasion (i.e., 83 to 67% invasion inhibition), relative to control samples. The parasite UAM37 contained both the FC27 and 3D7 alleles of MSP2 and all three alleles tested for MSP1, while UAS31 contained only 3D7 of MSP2 and RO33 of MSP1 (but not K1 or MAD20). When all patient plasma samples were tested against UAM37 and UAS31, there was a trend of decreased invasion (57.9%) compared to invasion in severe malaria cases (70% invasion) when plasma from uncomplicated malaria patients was used against UAS31, but the difference was not significant (Table 2). A subset of samples (four from patients with uncomplicated malaria and four from patients with severe malaria) was used to test whether the decreased parasite invasion was due mainly to an invasion-inhibitory or growth-inhibitory effect. We performed invasion inhibition assays with analysis of parasitemia using microscopy and flow cytometry at different time points (data not shown) and could see that a major part of the inhibition in our assay setup was found within the first 8 h; hence, we chose to refer to our assay as an invasion inhibition assay (IIA).

Correlation between different assays for measuring antibod-

ies and initial parasitemias. Both clinical \( P. falciparum \) isolates (UAM37 and UAS31) showed increased invasion in the presence of plasma from individuals with high initial parasitemias (Fig. 3), indicating a lack of inhibitory antibodies (for UAS31, \( R^2 = 0.22 \) and \( P = 0.0009 \); for UAM37, \( R^2 = 0.16 \) and \( P = 0.006 \)).

There was no correlation between initial parasitemias and the presence of either IgG or IgM with any of the tested antigens, as measured by ELISA, and no correlation between initial parasitemias and affinity of antibodies against the antigens tested.

Correlation between different aspects of measuring antibod-

ies. The most extensive analysis for comparison of methods for measurement of antibody responses in plasma was carried out for the MSP2-3D7 and MSP2-FC27 antigens, for which we had access to two different allelic families of the proteins. As expected (since the two alleles partly contain the same sequences), IgG and IgM ELISA results for both allelic families correlated well with each other (\( P \) values for all comparisons ranged from 0.02 to \(<0.0001\), and \( R^2 \) values ranged from 0.07 to 0.50, except for the comparison between IgM MSP2-3D7 and IgG MSP2-FC27, for which the \( P \) value was 0.1 and \( R^2 \) was 0.03). When MSP2 NH\(_4\)SCN ELISA results were compared to IgG ELISA results, significant positive correlations could be seen both for MSP2-3D7 NH\(_4\)SCN (\( R^2 = 0.36 \) and \( P = <0.0001 \)) and both MSP2-FC27 ELISA and MSP2-3D7 ELISA) and for MSP2-FC27 NH\(_4\)SCN (\( R^2 = 0.3 \) and \( P < 0.0001 \) against MSP2-FC27 ELISA; \( R^2 = 0.07 \) and \( P = 0.02 \) for MSP2-3D7 ELISA).

Between parasite invasion and presence of antibodies in IgG/ IgM ELISAs, a significant correlation could be seen for UAS31 between IIA and the IgG ELISA responses against MSP2-FC27 (\( R^2 = 0.20 \) and \( P = 0.0016 \)), for UAM37 against EBA175 (\( R^2 = 0.09 \) and \( P = 0.03 \)), and for both UAS31 and UAM37 against PrRh2 (UAS31, \( R^2 = 0.16 \) and \( P = 0.006 \); UAM37, \( R^2 = 0.14 \) and \( P = 0.01 \)), with higher levels of antibodies in samples with decreased parasite invasion. The best correlations were seen when IIA results were compared to IgG ELISAs for AMA1 for both isolates (UAS31, \( R^2 = 0.25 \) and \( P = 0.0003 \); UAM37, \( R^2 = 0.19 \) and \( P = 0.0023 \)) (Fig. 4). For AMA1, we could also see a correlation between high levels of IgG antibodies in ELISAs and high affinity in SPR (\( R^2 = 0.31 \) and \( P < 0.0001 \)) (Fig. 5).

For the other recombinant proteins used in IgG ELISAs (EBA140, EBA181, PrRh4, MSP2-3D7, and MSP1), no significant correlations could be seen with IIA results.
Correlation of antibody affinity (measured as $k_d \text{[s}^{-1}]$) by SPR in relation to anti-AMA1 IgG (measured by ELISA) in plasma from patients with uncomplicated or severe malaria ($R^2 = 0.31$ and $P < 0.0001$).

When IgG/IgM ELISA results were compared to each other, many of the IgG results correlated to each other. For example, AMA1 ELISA results correlated with ELISA results for MSP2-FC27, MSP2-3D7, EBA175, EBA181, and Rh2; EBA181 ELISA results correlated with ELISA results for MSP2-FC27, MSP2-3D7, AMA1, EBA175, Rh2, and Rh4.

In conclusion, many of the ELISA results correlated with each other and with NH$_4$SCN ELISAs, but when ELISAs were compared to IIA and antibody affinity results, AMA1 stood out as showing more correlations between methods than for other proteins.

**TABLE 3** MOI expressed as the number of clones in the patient samples for each analyzed gene

| Gene product and no. of clones | Uncomplicated malaria | Severe malaria |
|-------------------------------|-----------------------|---------------|
| CSP (0 clones)                | 0                     | 0             |
| CSP (1 clone)                 | 59.3                  | 58.6          |
| CSP ($\geq2$ clones)          | 40.7                  | 41.1          |
| GLURP (0 clones)              | 0                     | 0             |
| GLURP (1 clone)               | 55.6                  | 5.9           |
| GLURP ($\geq2$ clones)        | 44.4                  | 24.1          |
| MSP1-K1 (0 clones)            | 18.5                  | 27.6          |
| MSP1-K1 (1 clone)             | 40.7                  | 65.5          |
| MSP1-K1 ($\geq2$ clones)      | 40.7                  | 6.9           |
| MSP1-MAD20 (0 clones)         | 29.6                  | 34.5          |
| MSP1-MAD20 (1 clone)          | 29.6                  | 55.5          |
| MSP1-MAD20 ($\geq2$ clones)   | 40.7                  | 10.0          |
| MSP1-RO33 (0 clones)          | 0                     | 41.4          |
| MSP1-RO33 (1 clone)           | 29.6                  | 58.6          |
| MSP1-RO33 ($\geq2$ clones)    | 70.4                  | 0             |
| MSP2-FC27 (0 clones)          | 25.9                  | 31.0          |
| MSP2-FC27 (1 clone)           | 40.7                  | 44.8          |
| MSP2-FC27 ($\geq2$ clones)    | 33.3                  | 24.1          |
| MSP2-3D7/IC (0 clones)        | 3.7                   | 6.9           |
| MSP2-3D7/IC (1 clone)         | 51.9                  | 75.9          |
| MSP2-3D7/IC ($\geq2$ clones)  | 44.4                  | 17.2          |

FIG 4 Correlation of percent parasite invasion of two clinical *P. falciparum* isolates (UAS31 and UAM37) in the presence of plasma from patients in relation to anti-AMA1 IgG (measured by ELISA). Significant correlations were found both for UAS31 ($R^2 = 0.26$ and $P = 0.0003$) and for UAM37 ($R^2 = 0.19$ and $P = 0.002$).

In this study, antibody responses in plasma from Ugandan children with uncomplicated or severe *P. falciparum* malaria were...
tested against a panel of merozoite antigens. In the effort to find a vaccine against malaria, the goal is to achieve sterile immunity against malaria, but if this cannot be accomplished (something that so far has been difficult in spite of numerous trials), protection against severe malaria would be a great achievement in reducing mortality due to malaria. Indeed, it has been estimated that immunity against severe disease can be reached after a small number of infections (31, 72), indicating that reduction of the severe malaria burden by a vaccine is an achievable goal. However, to know which antigens are protective, we have to know which methods to use. A first step is to compare patients with uncomplicated and severe malaria to discern differences in antibody responses, and we have in this paper used a selection of methods, including ELISA, IIA, SPR, and NH\(_4\)SCN ELISA, in combination with studies of the parasites in the blood since no single method yet is strongly predictive of immunity (12, 17, 33, 34, 41, 43–45). ELISAs have been used for many years, but the ELISA is a static assay and may not be reflective of the function of the antibodies. SPR can offer studies of protein-protein interactions under flow, which ought to be more physiological than an ELISA. By using a combination of assays, we aimed to increase the understanding of the immunological response, with the goal of better prioritization of vaccine candidates.

We compared children with uncomplicated and severe malaria and found higher IgG levels in ELISAs in patients with uncomplicated malaria against a selection of merozoite antigens: EBA181, MSP2-3D7, MSP2-FC27, and AMA1. Earlier studies have shown associations between high levels of antibodies against several different merozoite antigens and protection against symptomatic malaria in general (even though severe malaria has usually not been studied specifically), while other studies consider high antibody levels more as a marker of exposure (22, 25, 28, 40, 72–75).

In our study, all patients lived in the same village, and the average age of the two groups (uncomplicated and severe malaria) were not significantly different, indicating similar exposure between the two groups. Many of the genetic host factors were probably also similar. The area has a high degree of endemicity, with an EIR of >1,500 infective bites per person per year (63, 64); most of the children had probably been exposed to malaria many times before they were included in the study, and no severe malaria cases occurred in children over 3 years of age during our study period. Despite the extreme transmission rate and presumed high levels of protection among patients in general, we could still detect some differences in antibodies between the two groups of patients.

When different ways of measuring antibodies are compared, the presence of antibodies against some antigens showed some interesting correlations with other properties such as parasite invasion and affinity results. For example, presence of antibodies against Rh2 and AMA1 in ELISAs correlated to invasion for both tested clinical isolates, and antibodies against AMA1 in ELISAs also correlated to affinity SPR results, indicating that this protein might be targeted by functionally important, high-affinity antibodies that could contribute to protection from severe malaria. Our findings are consistent with recent studies from Uganda (76, 77) that showed a strong association between antibodies against AMA1 and protection against malaria. Other antigens, such as MSP1, showed no difference in antibody levels between uncomplicated and severe malaria and did not show any correlations with invasion. Invasion of the merozoite into red blood cells is a process that takes only a few minutes, and antibodies probably need to be of high affinity to be able to exert their functions. Antibodies against an antigen like AMA1 might therefore turn out to be more important than other merozoite antigens in correlations with functional invasion assays. The presence of antibodies against AMA1 has previously been shown to be associated with a reduced incidence of malaria (22, 28), and it has also been shown that individuals living in areas where malaria is endemic have increased levels of anti-AMA1 antibodies, which can be strongly inhibitory (12, 78, 79). A general problem for many vaccine candidates is polymorphism, and this might be a hurdle also for AMA1. AMA1 has been shown to be a target of allele-specific immune responses (28, 80, 81), and positive selection could have produced diverse conformational epitopes to avoid recognition by antibodies. However, studies have shown that both conserved and strain-specific epitopes are targets of inhibitory antibodies (14, 80, 82, 83), and when multialele immunization was used in vaccine studies, the cross-reactivity of induced antibodies could be increased (84), which suggests a way to overcome the problem of polymorphism. It can also make a difference which form of protein is chosen to use in vaccine studies. When 262 individuals in Papua New Guinea were tested for the presence of antibodies against different allelic forms of AMA1, it was found that the D10 form contained most of the epitopes (14). This was the protein used in our studies. We have not tested whether the parasites in the study region contain D10, but since 100% of the individuals were positive in ELISAs, we assume that they have at some stage been exposed to this parasite.

MSP2 can be grouped into two main allelic families, either ICI/3D7 or FC27 (11, 85, 86). In our studies, we mainly used recombinant proteins from the 3D7 parasite for most of the antigens, but for MSP2 we included both the 3D7 and FC27 forms. When the parasites in the blood of the patients were analyzed by PCR, we could see that most patients had both MSP2-3D7 and MSP2-FC27 allelic variants. Absence or low levels of antibodies in some patients, therefore, cannot be explained by the idea that people in general have not been exposed to the parasites in this area. The presence of both alleles in Uganda has also been shown previously (87, 88).

Not many studies have investigated differences between uncomplicated and severe malaria for merozoite antigens; most of the studies have so far focused on surface antigens of the infected red blood cell, such as PfEMP1, or have used schizont extract (that includes both merozoite and infected red cell antigens) in these kinds of investigations (89–91). In one study using merozoite antigens comparing uncomplicated and severe malaria, it was found that antibodies against MSP1 were present at higher levels in patients that recently had uncomplicated malaria, whereas levels of antibodies against specific peptides of MSP1 were higher in patients that had severe malaria (92). For MSP1 it has also been shown that there are antibodies that can block cleavage of the protein, but human plasma also contains antibodies that can block the processing-inhibiting antibodies (93). This further shows the importance of using more functional assays to evaluate the total effect of different antibodies together.

In our study group, the levels of IgG antibodies against both MSP2-FC27 and MSP2-3D7 were significantly higher in uncomplicated malaria than in severe malaria. The IgG ELISA results correlated very well with those of the NH\(_4\)SCN ELISA, which is not surprising since the methods are very similar. MSP2 (full-length recombinant protein used in the assays) contains both con-
erved and variable domains, and there might be some overlap in antibody responses between the two allelic variants due to antibodies reacting against the same epitope. We could not see any major correlations in affinity of antibodies when SPR results and NH4SCN ELISAs were compared, which could be explained by the NH4SCN ELISA being a static method while SPR measures the interaction between antibody and antigen in real time under flow. Other antibodies might be of importance, and higher affinity might be needed for antibodies to remain bound when there is a constant flow in the system. Earlier studies have shown correlations between low levels of antibodies against MSP2 and increased risk of malaria (27). It has also been shown that antibodies against MSP2-3D7, but not against MSP2-FC27, have been associated with protection against malaria (94). In line with this, it is interesting that we consistently found a higher affinity of antibodies against the 3D7 allelic variant (than against FC27) when SPR was used. Part of the reason for this difference might be that the 3D7 allelic variant of the protein can form a structure that is more prone to forming high-affinity antibodies. MSP2 is considered to be an unstructured protein and might therefore adopt different conformations when different antibodies bind to the protein (10, 95). Another reason could be that the 3D7 allelic variant has a unique epitope that can trigger production of high-affinity antibodies. Other studies have also pointed toward MSP2-3D7 as being more important in protection against severe malaria (96, 97). When the parasites in the blood of the patients in this study were analyzed, more genotypes were found in uncomplicated malaria cases of MSP2-3D7 than in severe cases, but no significant differences were seen for MSP2-FC27. This might also point toward the 3D7 allele as being more important. For MSP1, it was only the K1 allele that reached significant differences, but the values for MAD20 (equivalent to 3D7) reached close to significant differences between uncomplicated and severe cases. It might be that once a good immune response has been mounted against the 3D7 allele, it is easier to survive the next episode of malaria. When the total MOI of the parasites was considered, uncomplicated malaria cases had higher numbers of genotypes. This has been shown before in areas with a high degree of endemicity (57) and is important information for vaccine trials, where it might be better to include several variants of a protein to achieve the best possible response. An alternative interpretation of the generally higher MOI for uncomplicated malaria cases is that tolerance to mult-clonal infections is just simply a measure of exposure, but since the MOI values did not correlate with age, we do not believe that this is the case.

To study the function of antibodies, we used IIAs. In previous studies where this method has been applied, mainly laboratory strains have been used, and it has been reported that antibodies that inhibit merozoite invasion could contribute to acquisition of natural immunity but may not necessarily confer definitive protective immunity against malaria; there are conflicting results as to whether the inhibitory effect of antibodies increases or decreases with age (31, 33, 35, 36, 38, 39, 98). In our study, we used two clinical P. falciparum isolates, UAM37 and UAS31, to investigate whether there is decreased invasion in the presence of plasma from patients with uncomplicated and severe malaria. For UAS31 we found a difference, with less invasion in the presence of plasma from uncomplicated cases of malaria, but this difference did not reach significance. This might be explained by the limited number of patients included in the study. However, there was more invasion in both clinical isolates in the presence of plasma from patients with high initial clinical parasitemia (severe malaria patients), suggesting the possible importance of functional assays in the evaluation of antibody responses. We also believe that the use of fresh clinical isolates in the IIAs might have added a substantial improvement to these assays as laboratory isolates are known to change after long-term growth in vitro (99). We could not see any correlation of IIA results with age, which might be explained by the limited age group of the children (all were under 3 years of age).

In conclusion, this study showed that both occurrence of certain allelic variants of parasites and a higher MOI as well as presence of elevated levels of antibodies with high affinity, especially against AMA1 but possibly also against MSP2, might be protective against developing severe malaria. It is of special interest that the only assay that presented a major correlation with initial parasitemia in the patients was the IIA using clinical isolates, which highlights the potential importance of using functional assays. Based on our results, we think that for future studies an ELISA is a good start to establish that antibodies are formed against a particular antigen. But in addition to ELISAs, it is important to select a combination of functional assays, such as IIAs and/or SPR assays, to better understand how immunity against malaria is developed and to be able to priorize antigens for vaccine trials. When merozoite antigens are considered as vaccine candidates, differences between uncomplicated and severe malaria should be considered in the evaluations as vaccines that reduce severe disease and mortality would be of major public health value.

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