Specificities of Antibodies to *Plasmodium falciparum* Merozoite Surface Protein (MSP)-19

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**Summary**

In a survey of children living in South Western Nigeria, plasma levels of anti-MSP19 antibodies were not associated with patent parasitemia. Anti-MSP19 antibody titres correlated positively with age, indicating that development of antibodies to MSP19 may depend on long-term exposure to parasites. Using competitive ELISA, 82% of the samples inhibited the binding of processing-inhibitory monoclonal antibodies (mAb) 12.8 and 12.10 to immobilized recombinant MSP19. The binding of mAb 12.8 in the presence of 18% of these samples was reduced to less than 10%. This suggests that these samples contain polyclonal antibodies that have a similar binding specificity to that of mAb 12.8, which recognizes an epitope located in the first epidermal growth factor domain of MSP19. Our data provide useful leads for the design of an MSP19-based vaccine.

**Introduction**

The *P. falciparum* merozoite surface protein (MSP)-1 is currently being evaluated as a vaccine candidate against malaria, a disease that annually affects more than 300 million people resulting in over a million deaths especially in children less than five years (WHO, 1997). MSP1 is synthesized as a 200-kDa glycoprophosphatidylinositol-anchored membrane protein precursor, which undergoes a two-stage proteolytic
processing reaction. The final secondary processing results in a 19 kDa C-terminal fragment (MSP1<sub>19</sub>) that is carried into the newly invaded erythrocyte by merozoites and is implicated in the invasion process (Blackman et al., 1990; Blackman and Holder, 1992). Sequence comparisons show that the MSP1<sub>19</sub> fragment contains two epidermal growth factor (EGF)-like domains that are cysteine-rich and relatively well conserved compared to other regions of MSP1 (Blackman et al., 1991).

Considerable evidence that antibodies to MSP1<sub>19</sub> can confer protection against malaria has been reported (Branch et al. 1998; Holder et al., 1999). These anti-MSP1<sub>19</sub> antibodies protect against erythrocyte invasion by inhibiting the secondary processing of MSP1<sub>42</sub> to MSP1<sub>19</sub>, their protective effect is critically dependent on the fine specificity of the antibodies. The effectiveness of an MSP1-based vaccine will therefore depend on the amount, the fine specificity and the affinity of the antibodies induced; the antibody isotype may also be important (Guevara Patiño et al., 1997; Holder et al., 1999; Nwuba et al., 2002). Two secondary processing-inhibitory monoclonal antibodies (mAb) have been described (Blackman et al., 1994): mAb 12.8 that recognises the first EGF-like domain of MSP1<sub>19</sub> and mAb 12.10 that requires both EGF domains for binding (McBride and Heidrich, 1987; Chappel and Holder, 1993; Burghaus and Holder, 1994). We have shown previously that a part of the natural immune response to MSP1 during malaria infection is antibody that competes and prevents the binding of these mAbs to MSP1<sub>19</sub>, suggesting similar epitope specificity (Guevara Patiño et al., 1997; Nwuba et al., 2002). In these studies, antibody-binding specificity was evaluated using a competition ELISA in which serum antibodies and monoclonal antibodies react competitively with immobilized recombinant MSP1<sub>19</sub>, essentially as described (Nwuba et al., 2002). Ninety-six-well polystyrene microtitre plates (Costar, Corning Inc., NY) were coated with 0.5 µg/ml recombinant MSP1<sub>19</sub> in coating buffer (0.5 M sodium carbonate, 0.035 M sodium bicarbonate, pH 9.6). Plasma samples were diluted serially and 100 µl incubated for 1 h at 37°C in each well. Bound antibodies were subsequently detected with horseradish peroxidase (HRP)-rabbit anti-human IgG conjugate (Dako, Denmark) and the reaction developed with ABTS/hydrogen peroxide substrate solution and measured at 650 nm as described by the manufacturers (KPL, MD). The binding of mAbs 12.8 and 12.10 to recombinant MSP1<sub>19</sub> in the presence of saturating concentrations of plasma antibodies was analysed by a competitive ELISA as described elsewhere (Burghaus et al., 1996; Guevara Patiño et al., 1999).

Knowledge of the fine specificity of antibodies to MSP1<sub>19</sub> is invaluable in order to predict their effect on MSP1 secondary processing. It is also important to determine the specific regions of MSP1 that elicit strong immune responses, particularly in the context of MSP1 sequence polymorphisms, as these regions could provide target epitopes for inclusion in a subunit vaccine. In this study, we have investigated the binding specificities to MSP1<sub>19</sub> of serum antibodies from malaria-exposed children, using a competitive ELISA and the processing-inhibitory mAbs 12.8 and 12.10. The relationship between anti-MSP1 antibody levels and patent parasitemia was also analysed.

Materials and Methods
Subjects and blood collection. The randomly selected subjects included 60 children (between 1 and 120 months) who were malaria-asymptomatic, from Idere, a rural area in South Western Nigeria. Blood samples were collected in October and November 1999. The children were recruited from the Expanded Programme on Immunization clinic. Malaria is hyperendemic in this region with a parasite prevalence of 75% among children during the rainy season from April to October and 55-60% during the dry season from November to March (Nwagwu et al., 1998). Two to five ml of blood was drawn by venipuncture into tubes containing 0.5 ml anticoagulant (0.12 M trisodium citrate). The plasma was removed by centrifugation and stored at -40°C. Thick and thin blood smears were prepared on slides in the field and stained with Giemsa reagent. Slides were examined for malaria parasites under a light microscope and the patent parasitemia expressed as parasites per microlitre of blood. The protocol used in this study was approved by the Ethical Committee, College of Medicine, University College Hospital, Ibadan, Nigeria.

Monoclonal antibodies. Murine mAbs 12.8 and 12.10 that bind to P. falciparum MSP1<sub>19</sub> have been described elsewhere (McBride and Heidrich, 1987; Blackman et al., 1990). The antibodies were biotinylated using EZ-link sulfo NHS-biotin as recommended (Pierce, IL).

Recombinant MSP1<sub>19</sub>. The expression of P. falciparum (Wellcome line) MSP1<sub>19</sub> from the pGEX-3X plasmid as a fusion protein with Schistosoma japonicum glutathione S-transferase (GST) has been described previously (Burghaus and Holder, 1994).

Enzyme-linked immunosorbent assay and competitive ELISA. An ELISA was used to determine the titre of antibodies to recombinant MSP1<sub>19</sub> essentially as described (Nwuba et al., 2002). Ninety-six-well polystyrene microtitre plates (Costar, Corning Inc., NY) were coated with 0.5 µg/ml recombinant MSP1<sub>19</sub> in coating buffer (0.5 M sodium carbonate, 0.035 M sodium bicarbonate, pH 9.6). Plasma samples were diluted serially and 100 µl incubated for 1 h at 37°C in each well. Bound antibodies were subsequently detected with horseradish peroxidase (HRP)-rabbit anti-human IgG conjugate (Dako, Denmark) and the reaction developed with ABTS/hydrogen peroxide substrate solution and measured at 650 nm as described by the manufacturers (KPL, MD). The binding of mAbs 12.8 and 12.10 to recombinant MSP1<sub>19</sub> in the presence of saturating concentrations of plasma antibodies was analysed by a competitive ELISA as described elsewhere (Burghaus et al., 1996; Guevara Patiño et al., 1999).

Results
Parasitemia. Patent parasitemia was determined by examination of Giemsa-stained blood smears using light microscopy. Of the 60 subjects examined, 21 (35% of the total) were negative for malaria parasites while the remaining 39 (65%) had patent parasitemia ranging between 28 and 50,000 parasites per ml of blood. The highest parasitemia was
observed in children less than 1-year old, with a marked peak in children between 4 and 6 months where a mean parasitemia of 5,507 parasites per μl of blood (Fig. 1) was detected.

Relationship between parasitemia, age and anti-MSP1 19 antibody titres. MSP1 19 antibody titre, expressed as the log reciprocal of the plasma dilution, increased with age (Fig. 2a and b) with a positive significant correlation (Pearson's product moment correlation coefficient \( r = 0.42; p < 0.001 \)). No relationship was found between the patent parasitemias detected during the survey and the anti-MSP1 19 antibody titres (Fig. 3). The negative correlation (Pearson's correlation coefficient \( r = -0.059 \)) was not significant (\( p = 0.65 \)).

Competition of plasma antibodies with processing-inhibitory mAbs for binding to MSP1 19. To define the regions of MSP1 19 recognized by plasma antibodies, the plasma samples were examined in a competitive ELISA using biotinylated mAbs 12.8 and 12.10, which bind to an epitope on the first EGF domain and an epitope formed from both EGF domains, respectively. Plasma antibodies that bind to similar regions of MSP1 19 as mAbs 12.8 and 12.10 would be expected to block the binding of these mAbs to MSP1 19, thus reducing by competition the amount of bound biotinylated antibody.

The binding of biotinylated mAbs 12.8 and 12.10 to recombinant MSP1 19 was reduced to less than 70% with 49 of the 60 plasma samples (81.7% of the total samples) in competition ELISA (data not shown). Generally, samples that competed with mAb 12.8 also competed with mAb 12.10; however competition was not a function of the overall anti-MSP1 19 antibody titre of each plasma sample (data not shown). Eleven samples (18.3% of the total) reduced the binding of mAb 12.8, but not
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Figure 4. Eleven of the sixty plasma samples reduced the binding of mAb 12.8 to recombinant MSP19 to 10% or less of the binding in the control sample (no Ab), indicating that they share the same epitope specificity with mAb 12.8. The binding of mAb 12.8 and the effect of adding the plasma samples were measured by a competitive ELISA. In this assay, 96-well ELISA plates pre-coated with recombinant MSP19 were incubated with plasma before addition of biotinylated mAb. Control assays were as follows: no Ab, labelled antibody bound in the absence of plasma; mAb, labelled antibody bound in the presence of competitor mAb. Bound mAb was detected by a streptavidin-horse radish peroxidase conjugate using OPD substrate and measuring the reaction product at 650 nm. The binding of biotinylated mAb is expressed as a percentage of the amount bound in the absence of competitor. All experiments were performed in triplicate. Open bars, binding of mAb 12.8; black bars, binding of mAb 12.10.

mAb 12.10, to less than 10% of mAb binding in the absence of competitor (Fig. 4). The marked competitive effect of these plasma samples suggests the presence of antibodies to the first EGF domain of MSP19. As shown in Fig. 5, the binding of both mAbs was greater than 70% in the presence of 11 plasma samples. Interestingly, two of these samples (21D4 and 21D44), both with detectable anti-MSPl9 antibodies, had no effect on the binding of either mAb 12.8 or 12.10. Presumably, these plasma samples contain antibodies that recognize epitopes on MSP19 distinct from those of mAb 12.8 and mAb 12.10.

Discussion

Antibodies to the cysteine-rich MSP19 fragment have been shown to have a protective effect against malaria in rodents and humans (Holder et al., 1999). Recent studies indicate that the protective anti-P. falciparum MSP19 antibodies inhibit MSP1 secondary processing and erythrocyte invasion, and the fine specificity of binding is crucial (Guevara Patino et al., 1997; Holder et al., 1999). We have observed that humans naturally exposed to malaria do produce processing-inhibitory antibodies specific for the first EGF domain of MSP19 (Nwuba et al., 2002). These observations suggest that in addition to measuring the total anti-MSPl9 titre in surveys of antibody responses, it is important to determine the specificities of the polyclonal antibodies even if their processing-inhibitory activity is unknown, since this information would help define the epitopes recognized by these antibodies.

In this study, we have investigated the specificities of antibodies to MSP19 in children living in Idere, a region of South Western Nigeria where malaria is hyperendemic. The results show that the antibody response of malaria-exposed children to the C-terminal MSP19 is in part specifically targeted to the first EGF domain as judged by the ability of the antibodies to compete with mAb 12.8 for binding to MSP19. Furthermore, other plasma antibodies competed with mAb 12.10, indicating that their specificity, like mAb 12.10, may comprise both EGF domains of MSP19 (Chappel and Holder, 1993). The ability of the plasma antibodies to reduce the binding of the mAbs in the competition ELISA was not dependent on the overall anti-MSPl9 titre, but is presumably a function of their fine specificity. No association was detected between patent parasitemia and the prevalence of these EGF-specific antibodies or the total anti-MSPl9 antibody levels.

Although we found no association between anti-MSPl9 levels and
The fine specificity of antibodies to MSP1 is crucial in determining whether they inhibit erythrocyte invasion by inhibiting MSP1 secondary processing. Thus, immuno-epidemiological studies that characterise the epitope specificities of anti-MSP1 antibodies provide valuable information for the development of an MSP1-based malaria vaccine.


