

Antibodies Inhibit the Protease-mediated Processing of a Malaria Merozoite Surface Protein

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Summary

When merozoites of the malaria parasite *Plasmodium falciparum* are released from infected erythrocytes and invade new red cells, a component of a protein complex derived from the merozoite surface protein 1 (MSP-1) precursor undergoes a single proteolytic cleavage known as secondary processing. This releases the complex from the parasite surface, except for a small membrane-bound fragment consisting of two epidermal growth factor (EGF)-like domains, which is the only part of MSP-1 to be carried into invaded erythrocytes. We report that, of a group of monoclonal antibodies specific for epitopes within the EGF-like domains, some interfere with secondary processing whereas others do not. Those that most effectively inhibit processing have previously been shown to prevent invasion. Other antibodies, some of which can block this inhibition, not only do not prevent invasion but are carried into the host cell bound to the merozoite surface. These observations unequivocally demonstrate that the binding of antibody to the COOH-terminal region of MSP-1 on the merozoite surface may not be sufficient to prevent erythrocyte invasion, and show that the interaction of different antibodies with adjacent epitopes within the EGF-like domains of MSP-1 can have distinct biochemical effects on the molecule. Inhibition of MSP-1 processing on merozoites may be a mechanism by which protective antibodies interrupt the asexual cycle of the malaria parasite.

The blood-stage malaria parasite replicates within host erythrocytes. At periodic intervals, parasitized cells rupture to release infectious merozoite progeny which rapidly reinvade fresh erythrocytes. The initial interaction between merozoite and red cell involves ligands on the merozoite surface (1), and it is widely documented that antibodies to merozoite surface proteins (MSPs) can interfere with parasite growth in vitro (2–9) and in vivo (10–12). In some cases these antibodies act by directly agglutinating released merozoites (7). However, in the majority of cases reported, the mechanisms involved are unknown.

A major component of the merozoite surface is a protein complex containing proteolytic fragments of the MSP-1 precursor (13). After merozoite release, a secondary processing event occurs in which the membrane-bound 42-kD component (MSP-1₄₂) of this complex is subjected to a single proteolytic cleavage (14, 15). The NH₂-terminal, 33-kD cleavage product (MSP-1₃₃) is shed with the remainder of the complex (16), whereas the COOH-terminal, 19-kD fragment (MSP-1₁₉), which consists of two epidermal growth factor (EGF)-like domains, remains on the merozoite (2, 14) (see Fig. 1). Secondary processing goes to completion when a merozoite successfully invades an erythrocyte (2, 17). Processing also takes place in free merozoites isolated from cul-

ture, allowing the development of an assay which we have used to partially characterize the protease responsible (16, 17). We have postulated that secondary processing may be an essential step in erythrocyte invasion, and were therefore interested in specific inhibitors of the proteolysis.

In this study, a group of IgG mAbs reactive with epitopes within the EGF-like domains of MSP-1 was screened for their ability to interfere with secondary processing.

Materials and Methods

Parasites. *Plasmodium falciparum*, strain FCB-1 and clone T9/96, was maintained in culture and synchronized as described previously (16, 17).

Antibodies. mAbs 12.8, 12.10, 7.5, and 2.2 have been described previously (2, 13), as have mAbs 111.4, 111.2, and 89.1 (18, 19). mAbs 1E1, 2F10, and 12D11 were produced recently in this laboratory (20). With the exception of mAb 89.1, all the antibodies recognize disulphide-constrained epitopes within the EGF-like domains of MSP-1 (see Fig. 1) as shown by their reactivity on Western blots with native (21) or recombinant (20, 22) MSP-1₁₉ in a nonreduced but not a reduced form. All mAbs were purified by affinity chromatography on protein A or protein G-Sepharose, and dialyzed exhaustively against PBS, pH 7.2, containing 1 mM CaCl₂ and 1 mM MgCl₂ (PBS Ca/Mg) before use.

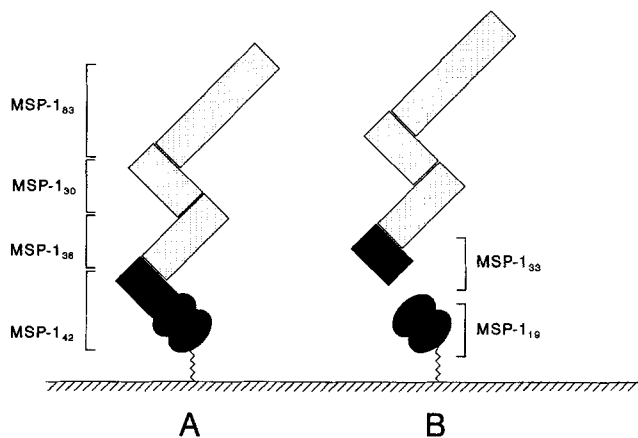


Figure 1. Schematic showing secondary processing of the *P. falciparum* MSP-1. (A) MSP-1 is present on the newly released merozoite surface as a complex of four polypeptides of ~83, 30, 38, and 42 kD (*MSP-1₈₃*, *MSP-1₃₀*, *MSP-1₃₈*, and *MSP-1₄₂*). *MSP-1₄₂* (derived from the COOH terminus of the precursor; shaded) is membrane bound through a glycosyl phosphatidylinositol anchor. (B) At or just before erythrocyte invasion, cleavage of *MSP-1₄₂* between Leu₁₆₃₀ and Asn₁₆₃₁ (numbering based on the deduced amino acid sequence of the complete MSP-1 gene; 15) produces *MSP-1₃₃* and *MSP-1₁₉* and releases the complex from the merozoite surface. *MSP-1₁₉* is represented as two EGF-like modules. A quantitative analysis of the processing (17) has shown that *MSP-1₄₂* is stoichiometrically converted to *MSP-1₃₃*. All the mAbs used in this study bind epitopes within *MSP-1₁₉*, except mAb 89.1, which recognizes an epitope on *MSP-1₈₃* (16, 19).

Production of a rabbit antiserum against a recombinant protein corresponding to the NH₂-terminal region of *MSP-1₄₂* has been described previously (17). The antiserum recognizes both *MSP-1₄₂* and *MSP-1₃₃*, but not *MSP-1₁₉*.

Merozoite Production. Mature FCB-1 schizonts were enriched from synchronous cultures by flotation on Plasmagel, and naturally released merozoites were isolated by membrane filtration as previously described (17, 23). Giemsa-stained smears of the merozoite preparations consistently indicated the complete absence of contaminating schizonts or uninfected erythrocytes.

Assays of MSP-1 Secondary Processing. Secondary processing of *MSP-1* in preparations of merozoites, and the effects of anti-*MSP-1*

mAbs on it, was assayed using the immunoblot method described previously (16, 17). Briefly, freshly isolated merozoites were washed in ice-cold PBS Ca/Mg, divided on ice into aliquots containing ~2 × 10⁹ merozoites each, and supplemented with either buffer only or purified mAb to a final antibody concentration of 400 μg ml⁻¹. One aliquot supplemented with buffer only was immediately solubilized in SDS; all other samples were incubated at 37°C for 1 h to allow secondary processing to occur before solubilization. All samples were then subjected to SDS-PAGE under non-reducing conditions on a 12.5% gel, electrophoretically transferred to nitrocellulose, and the blot probed with the rabbit antiserum to *MSP-1₃₃*.

The above methodology was modified to assess the ability of antibodies which did not affect processing, to competitively block the activity of those that did. Aliquots of washed merozoites on ice were supplemented with either buffer only or purified mAb to a final antibody concentration of 400 μg ml⁻¹. After a 15-min incubation (pretreatment) on ice, samples were further supplemented with processing inhibitory mAbs 12.8 or 12.10, or 1E1 (400 μg ml⁻¹ final concentration), placed at 37°C for 1 h, then SDS-solubilized and analyzed by immunoblot as described above.

Merozoite Invasion Inhibition Tests. These were carried out as described previously (2).

Analysis of Newly Parasitized Erythrocytes for the Presence of Antibodies Carried in on Invading Merozoites. Mature schizonts were enriched from synchronous cultures by flotation on Plasmagel (17) or centrifugation over 63% isotonic Percoll (21) and added to fresh erythrocytes to obtain a final parasitaemia of 10%. The cells were resuspended to a 5% hematocrit in culture medium, divided into aliquots, then supplemented with either culture medium only or purified mAbs, previously dialyzed against culture medium. Final mAb concentration in these cultures was 500 μg ml⁻¹. After incubation at 37°C for 4 h to allow merozoite release and erythrocyte invasion, residual schizonts were removed by three cycles of centrifugation over 67.5% isotonic Percoll (2). The resulting preparations, containing only uninfected erythrocytes and newly parasitized cells, were washed twice in medium containing 5% FCS, smeared onto slides, air-dried, and acetone fixed. Intracellular, parasite-associated antibody was visualized by incubation of fixed preparations with a FITC-conjugated goat anti-mouse IgG antibody (Sigma Chemical Co., St. Louis, MO) diluted 1:100 in PBS containing 5% vol/vol FCS. Slides were counterstained with 0.01% wt/vol Evans blue in PBS, and examined by fluorescence microscopy.

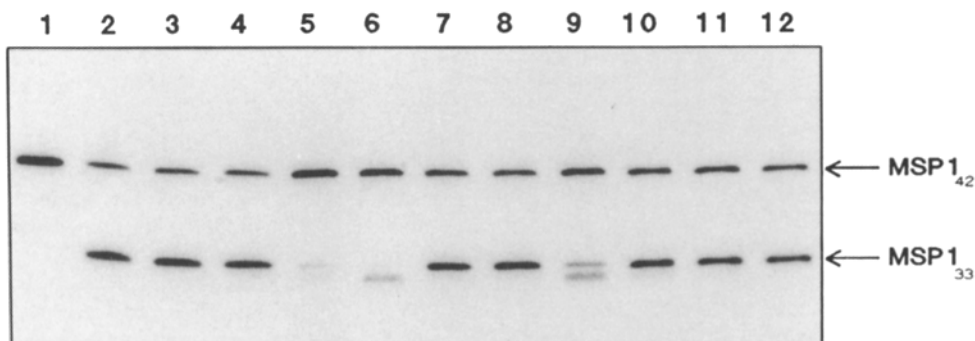


Figure 2. Secondary processing of *MSP-1* is inhibited by some but not all mAbs specific to epitopes within the EGF-like domains. Shown is processing of *MSP-1₄₂* in isolated, freshly washed merozoites (lane 1); after further incubation of the merozoites at 37°C in the absence of added antibody (lane 2); or in the presence of purified mAbs 2.2 (lane 3), 7.5 (lane 4), 12.8 (lane 5), 12.10 (lane 6), 2F10 (lane 7), 12D11 (lane 8), 1E1 (lane 9), 111.4 (lane 10), 111.2 (lane 11), and 89.1 (lane 12). Secondary processing is indicated by the appearance of a band corresponding to *MSP-1₃₃*, and a concomitant decrease in the intensity of the *MSP-1₄₂* band.

Results and Discussion

All the anti-MSP-1₁₉ mAbs used in this study bind to the surface of unfixed merozoites, and to acetone-permeabilized newly invaded (ring-stage) parasites as ascertained by indirect immunofluorescence assays (data not shown). Fig. 2 shows that only three of the mAbs inhibited secondary processing on merozoites. All three mAbs also induced some abnormal processing, resulting in a slightly truncated form of MSP-1₃₃ in addition to or instead of the usual product. Inhibition was dose dependent and detectable at antibody concentrations as low as 40 $\mu\text{g ml}^{-1}$ in the case of mAbs 12.8 and 12.10; mAb 1E1 was 10-fold less potent (data not shown). To confirm that the inhibition was a direct effect of antibody

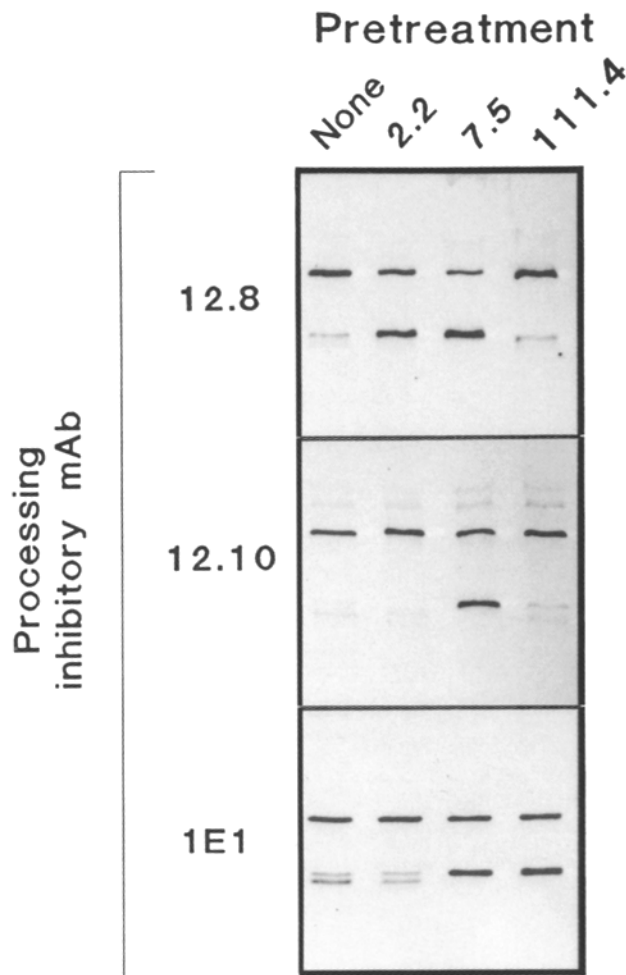


Figure 3. mAbs which do not inhibit MSP-1 processing can competitively block the activity of mAbs which do inhibit processing. Merozoites, either untreated or pretreated on ice with mAbs 2.2, 7.5, and 111.4, were incubated at 37°C in the presence of the processing inhibitory mAbs 12.8, 12.10, or 1E1 to assess the effects of pretreatment on processing inhibition. Shown are Western blots indicating that: the processing inhibitory effects of mAb 12.8 were blocked by pretreatment with mAbs 2.2 and 7.5, but not 111.4; the inhibitory effects of mAb 1E1 were blocked only by mAb 7.5; and the inhibitory effects of mAb 12.10 were blocked only by mAb 7.5 and 111.4. These results were completely reproducible in six separate experiments.

binding to MSP-1, we made use of the earlier observations of Wilson et al. (24). These authors used a two-site RIA to show that the binding of mAb 12.8 to purified MSP-1 was blocked when any one of mAbs 2.2, 7.5, or 12.10 occupied their epitopes. Similarly, binding of mAb 12.10 was blocked by the binding of mAbs 12.8 and 7.5, but not by

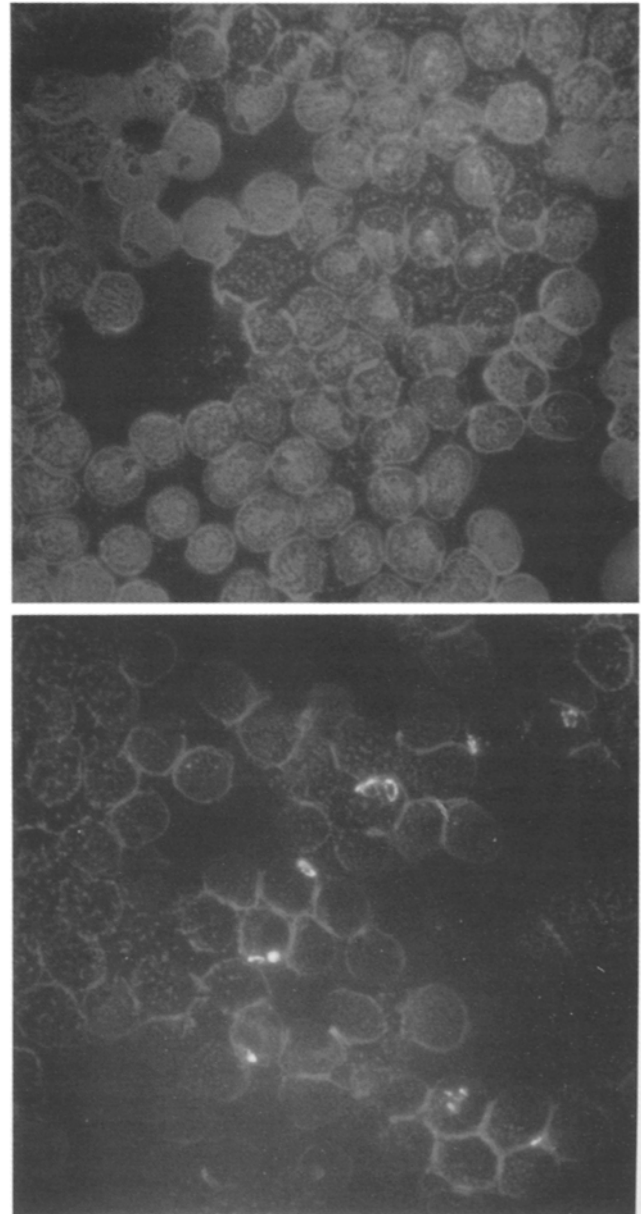


Figure 4. Antibodies reactive with MSP-1₁₉ are bound by merozoites and carried into the invaded erythrocyte. Direct immunofluorescence analysis of erythrocytes invaded by FCB-1 merozoites in the presence of (*top*) no added antibody or (*bottom*) mAb 12D11. Fluorescent intracellular ring stage parasites are clearly evident in the bottom panel. Identical results were obtained with mAbs 111.4, 111.2, 2F10, and 1E1, but not mAb 89.1 (not shown). T9/96 ring stage parasites formed in the presence of mAbs 111.4 and 12D11 did not possess detectable bound antibody (not shown).

mAb 2.2. We found that the inhibitory effects of antibodies on processing could be specifically blocked in a manner consistent with these data. Fig. 3 shows that pretreatment of merozoites with mAbs 2.2 or 7.5 blocked the inhibitory activity of mAb 12.8. In the case of mAb 12.10, pretreatment with mAb 7.5, but not mAb 2.2, blocked its activity. Pretreatment with mAb 111.4 had no effect on the activity of mAbs 12.8 and 12.10, but did block the activity of mAb 1E1, indicating that 1E1 binds an epitope distinct from those recognized by mAbs 12.8 and 12.10. The effects of all three mAbs were, however, blocked by pretreatment with mAb 7.5, suggesting that the three epitopes are adjacent.

We have previously reported that mAbs 12.8 and 12.10 prevent erythrocyte invasion when added to *in vitro* cultures of *P. falciparum* (2). When purified mAbs 111.4, 111.2, 12D11, 2F10, and 1E1 were assayed for their ability to inhibit invasion in cultures of parasite strains FCB-1 or T9/96, no inhibition was seen at antibody concentrations of ≤ 2 mg ml⁻¹. Since it seemed unlikely that under these conditions invading merozoites were completely evading the antibody, we analyzed intracellular ring-stage parasites from these cultures for the presence of antibody. Newly parasitized cells were acetone fixed and probed with a FITC-conjugated anti-mouse IgG. Fig. 4 shows that a strong rim of fluorescence was detected associated with the intracellular parasites. No fluorescence was seen with T9/96 rings produced in the presence of mAbs 111.4 and 12D11 (data not shown). These mAbs recognize epitopes not present on the MSP-1 allele expressed by T9/96 (22). Furthermore, parasites allowed to undergo invasion in the presence of mAb 89.1, which recognizes a conserved epitope on a fragment of MSP-1 which is shed after

secondary processing (16, and Fig. 1), did not show fluorescence. The antibody associated with the intracellular rings had bound specifically to the invading merozoite surface, and was not the result of nonspecific incorporation of fluid phase antibody into the parasitophorous vacuole during invasion.

These results prove that malaria merozoites can successfully invade erythrocytes despite the presence of surface-bound antibody, and suggest that for such antibody to prevent invasion it must mediate some additional effects deleterious to the parasite. We propose that inhibition of secondary processing of MSP-1 is one such effect. This is the first demonstration of a parasite enzymic activity susceptible to modification by antibodies which prevent erythrocyte invasion. It is also the first evidence that the fine specificity of antibodies directed towards a small region of MSP-1 can determine their function. The observation that antibodies which do not affect processing can block the inhibitory activity of other antibodies could explain the paradoxical findings that individuals in malaria-endemic areas may support significant parasitemias despite the presence of high plasma levels of naturally acquired antibodies to MSP-1 (25); the relative concentrations and affinities of processing inhibitory and blocking antibodies may determine the overall effect on cleavage of the MSP-1 complex from the surface of released merozoites, and in turn the ability of the parasites to invade erythrocytes. This hypothesis can now be tested experimentally using the antibodies and the processing assay described here. MSP-1 is a promising candidate for inclusion in a subunit malaria vaccine (15). The assay described may therefore provide a quantifiable prediction of resistance to blood-stage infection, which will be of use in malaria vaccine trials with MSP-1.

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