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Mode of Action of Invasion-Inhibitory Antibodies Directed against Apical Membrane Antigen 1 of *Plasmodium falciparum*

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Antibodies against apical membrane antigen 1 (AMA-1) of *Plasmodium falciparum* inhibit merozoite invasion into erythrocytes. Invasion-inhibitory polyclonal AMA-1 antibodies inhibit secondary proteolytic processing and surface redistribution of AMA-1 on merozoites. We present evidence supporting inhibition of processing and redistribution as probable causes of inhibition of invasion by polyclonal antibodies. Polyclonal anti-AMA-1 was much more inhibitory than monoclonal antibody (MAb) 4G2dc1 in an invasion assay. Although both polyclonal and monoclonal immunoglobulin G (IgG) inhibited secondary processing of the 66-kDa form of AMA-1, only polyclonal IgG caused its anomalous processing, inhibited its redistribution, and cross-linked soluble forms of AMA-1 on merozoites. Moreover, Fab fragments of polyclonal IgG that fail to cross-link did not show the enhancement of inhibitory effect over intact IgG, as observed in the case of Fab fragments of MAb 4G2dc1. We propose that although blocking of biologically important sites is a common direct mode of action of anti-AMA-1 antibodies, blocking of AMA-1 secondary processing and redistribution are additional indirect inhibitory mechanisms by which polyclonal IgG inhibits invasion. We also report a processing inhibition assay that uses a C-terminal AMA-1-specific MAb, 28G2dc1, to detect merozoite-bound remnants of processing (~20 kDa from normal processing to 48 and 44 kDa and ~10 kDa from anomalous processing to a 52-kDa soluble form of AMA-1). The ratio of intensity of 10-kDa bands to the sum of 10- and 20-kDa bands was positively correlated with inhibition of invasion by polyclonal antibodies. This assay may serve as an important immunochemical correlate for inhibition of invasion.

The merozoite stage of the malaria-causing parasite, *Plasmodium*, is highly specialized for invasion of human erythrocytes, and proteins that assist invasion are prime targets for clinical evaluation as malaria vaccines (4). Apical membrane antigen 1 (AMA-1) is one such invasion determinant on merozoites. Bivalent monoclonal (MAb) and polyclonal antibodies as well as their respective monovalent Fab fragments inhibit invasion of *Plasmodium* merozoites into erythrocytes (5, 11, 16, 17, 25), and immunization with recombinant AMA-1 protects against live parasite challenge in animal models of malaria (1, 24). AMA-1 of *Plasmodium falciparum* is first detectable as a *trans*-membrane protein of 83 kDa (PfAMA-1$_{83}$) in the apical complex of developing merozoites (21, 22). At the time of schizont rupture, a short N-terminal prosequence of PfAMA-1$_{83}$ is cleaved, resulting in a 66-kDa form (PfAMA-1$_{66}$) (13, 14, 21). PfAMA-1$_{66}$ translocates from the apical complex and redistributes on the merozoite surface (10, 21). Once on the surface a secondary proteolytic processing event cleaves PfAMA-1$_{66}$, shedding a 48-kDa soluble form of AMA-1 (13, 14, 21). A proportion of the shed 48-kDa molecules contain an intramolecular cleavage, with the two resulting fragments held together by a disulfide bond (14). Both 48-kDa soluble forms of AMA-1 comigrate on nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); however, on a reduced gel the nicked fragment migrates at 44 kDa

(PfAMA-1$_{44}$) (14). The biological significance of secondary processing of AMA-1 is not clear; however, the timing of these events suggests that processing and redistribution of AMA-1 may be necessary for host cell invasion (10, 13, 14, 21).

We have previously reported that invasion-inhibitory polyclonal anti-AMA-1 inhibited the secondary processing of PfAMA-1$_{66}$ to PfAMA-1$_{48+44}$ (6). Polyclonal antibodies also caused cross-linking of soluble PfAMA-1$_{48+44}$ forms on the merozoite surface, formation of a novel 52-kDa form (PfAMA-1$_{52}$), and inhibition of circumferential redistribution of AMA-1 on the merozoite surface. Based on our first observations with immune reagents and protease inhibitors, we had proposed that secondary processing of AMA-1 was essential for invasion and that an assay for measuring the processing-inhibitory activity of AMA-1-specific immune reagents may be predictive of their ability to inhibit invasion of merozoites in vitro (6).

In this report invasion inhibition, processing inhibition, and immunofluorescence assays were used to study the effects of bivalent immunoglobulin G (IgG) and monovalent Fab on merozoite invasion, AMA-1 processing, and AMA-1 redistribution. The Western blotting protocol used in this study was modified to include biotin-labeled MAb 28G2dc1 directed against the C terminus of *P. falciparum* AMA-1 (21) to enable the detection of merozoite-associated, low-molecular-weight products of AMA-1 processing. This study aims to establish the mechanism of antibody-mediated invasion inhibition, specifically to determine if inhibition of processing and redistribution contributes towards invasion inhibition.

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MATERIALS AND METHODS

Antibodies. Rabbit antibodies were raised against recombinant AMA-1 ectodomain (residues 83_{Gly} to 531_{Glu}) of *P. falciparum* strain 3D7 (5) or an identical AMA-1 construct derived from the FVO strain of *P. falciparum* (S. Dutta et al., unpublished data). Sera from rabbits immunized with a mixture of 3D7 and FVO AMA-1 proteins were also used. The sera were raised using either Montanide ISA720 (Seppic Inc., Paris, France) or AS02A adjuvants (Glaxo-SmithKline, Rixensaart, Belgium). MAb 4G2dc1 recognizes a conformational epitope on the PfAMA-1 ectodomain (18), MAb 28G2dc1 recognizes a highly conserved region on the C terminus, and MAb 58F8dc1 recognizes an N-terminal region of PfAMA-1 (21). MAbs were produced and purified by Strategic BioSolutions Inc. (Newark, Del.). All sera were heat inactivated at 56°C for 30 min prior to use. Serum IgG was purified as per the manufacturer's instructions using a 1-ml protein G column (Amersham, Uppsala, Sweden). Recombinant AMA-1 protein (5 mg/ml) was covalently linked to Cynogen Bromide Sepharose 4B (Amersham) according to the manufacturer's instructions. Five milligrams of purified polyclonal IgG was passed over a 1-ml CNBr–AMA-1 column, which was washed with 10 ml of phosphate-buffered saline (PBS), and antibodies were eluted using a low-pH IgG elution buffer (Pierce, Rockford, Ill.) and neutralized with 1 M Tris, pH 8.0. The elution and wash samples were monitored in an enzyme-linked immunosorbent assay (ELISA) for anti-AMA-1 antibodies. A portion of this affinity-purified anti-AMA-1 IgG along with MAbs 4G2dc1 and 58F8dc1 were digested with immobilized papain (ImmunoPure Fab kit; Pierce) overnight at 37°C to obtain their respective Fab fragments. Complete digestion of IgG was confirmed by gel electrophoresis. The Fc portion was not separated from the Fab fragments to avoid loss of antibody. Instead, total protein before and after papain treatment was estimated by using the Bio-Rad protein assay reagent (Richmond, Calif.). All purified antibody preparations were dialyzed against PBS prior to use, and samples were concentrated using a 10-kDa-cutoff Centricon concentrator (Amicon, Bedford, Mass.).

Parasites. *P. falciparum* clone 3D7 cultures were maintained and synchronized by the temperature cycling method (8). Mid-stage schizonts were purified by the Percoll-alanine method (15), and preparations of >90% pure, ~8-nucleated schizonts were used in the processing inhibition assay.

Processing inhibition assay. The processing inhibition assay was performed essentially as described previously (6). Briefly, 20 μl of an appropriate dilution of the antibody reagent was added to 80 μl of $10^7 \cdot ml^{-1}$ purified schizonts of the 3D7 strain in a 48-well plate. The plate was incubated at 37°C until >90% schizonts had ruptured. The resulting merozoites were harvested by centrifugation and washed once with PBS. The parasite pellet was suspended in SDS-PAGE sample buffer, and the proteins were separated on a nonreducing SDS-PAGE. AMA-1-specific bands were immuno-stained on a Western blot essentially as described previously (6), except biotinylated MAb 28G2dc1 (against the C terminus of AMA-1) and biotinylated polyclonal rabbit anti-AMA-1 IgG against the ectodomain (2 mg/ml and 1:1,000 dilution each) were used together as a primary antibody mix. Blots were scanned, and densitometric analysis was carried out as reported previously (6).

Invasion inhibition assay, ELISA, and indirect immunofluorescence assay. An invasion inhibition assay was performed under suspension conditions with the initial conditions of 4% hematocrit uninfected erythrocytes and ~0.2 to 0.5% schizonts of *P. falciparum* 3D7 strain using methodologies described previously (5, 8, 9). Aliquots of freshly invaded erythrocytes were stained with Giemsa to determine if the merozoites that failed to invade in the presence of anti-AMA-1 were agglutinated. The ELISA was performed using recombinant 3D7 AMA-1 coated on plates, essentially as described previously (5). Apical or circumferential localization of AMA-1 on merozoites released in the presence of immune or protease inhibitor reagents was determined using dual immuno-staining with rabbit anti-AMA-1 polyclonal antibodies and mouse anti-merozoite surface protein 1 (MSP-1) MAb 5.2 essentially as described previously (6).

RESULTS

Specificity of the AMA-1 processing assay. To confirm the specificity of the modified Western blot protocol, the synthesis and processing of PfAMA-1 on merozoites was followed in the presence of anti-3D7 AMA-1 antibodies. Schizonts were incubated with rabbit serum, adjuvant control (1:10 dilution, noninhibitory in an invasion assay), immune serum (1:2,500 dilution, noninhibitory), or immune serum (1:10 dilution, ~90% inhibitory). Parasites were collected at five time points corre-
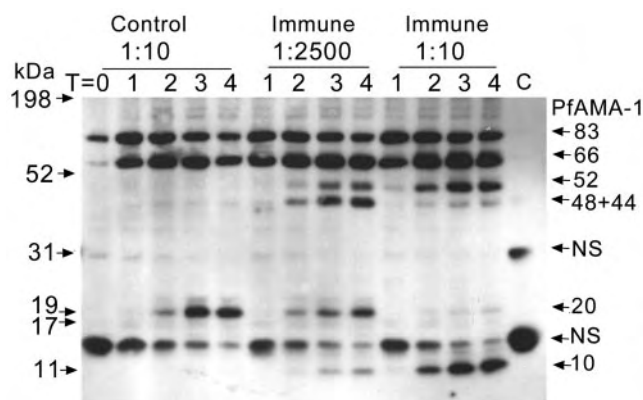

FIG. 1. Time course processing inhibition assay. *P. falciparum* 3D7 schizonts were incubated at 37°C with either adjuvant control (1:10 dilution) or postimmune serum at a 1:2,500 or 1:10 dilution, and samples were harvested at four time points as follows: $T_0 = 0$ h, 0% rupture; $T_1 = 3$ h, ~30%; $T_2 = 4$ h, ~50%; $T_3 = 5.5$ h, ~70%; $T_4 = 7$ h, >90% rupture. The samples were run on SDS-PAGE and Western blotted. The blot was probed with a mixture of two biotinylated primary antibodies: rabbit polyclonal anti-AMA-1 IgG and rat MAb 28G8dc1. Positions of molecular mass marker bands (Multimark; Invitrogen) are represented in kilodaltons on the left, and positions of the PfAMA-1-specific bands are shown on the right. Lane C had an equivalent number of uninfected erythrocytes incubated with a 1:10 dilution of the immune serum. NS, nonspecific bands derived from erythrocyte or serum components.

sponding to a desired percentage of schizont rupture: $T_0 = 0$ h, 0% rupture; $T_1 = 3$ h, ~30%; $T_2 = 4$ h, ~50%; $T_3 = 5.5$ h, ~70%; $T_4 = 7$ h, >90% rupture. The resulting parasite aliquots were analyzed by Western blotting (Fig. 1). The expected stage-specific synthesis and processing profiles of the PfAMA-1_{83}, PfAMA-1_{66}, cross-linked PfAMA-1_{52}, and cross-linked PfAMA-1_{48+44} bands were observed in the presence and absence of anti-AMA-1 (6). On this Western blot, two additional bands, one at ~20 kDa and another at ~10 kDa, were also detected. Both the ~20- and ~10-kDa bands reacted only with the C terminus MAb 28G2dc1 and showed no reaction with polyclonal antibodies against the ectodomain (data not shown). The MAb 28G2dc1 has been shown to react with PfAMA-1 on ring stages of *P. falciparum* by immunofluorescence assay (21), and it also immunoprecipitates a C-terminal 22/24-kDa doublet from the ring stages (13). The ~20-kDa AMA-1-specific band (PfAMA-1_{20}) represents the merozoite-borne remnant of the normal PfAMA-1_{66} processing to PfAMA-1_{48+44}, while the ~10-kDa band (PfAMA-1_{10}) represents the merozoite-borne remnant of the PfAMA-1_{52} cleavage. A ~15- and a ~30-kDa band were also observed on this Western blot. The ~15-kDa band was shown to be erythrocyte derived, as it was also detected in lane C, which had an equivalent number of uninfected erythrocytes incubated with a 1:10 dilution of immune serum, and the ~30-kDa band was found to be antibody derived. Both the ~15- and ~30-kDa bands are labeled as nonspecific in the figures.

We had previously suggested that PfAMA-1_{52} may be further cleaved to PfAMA-1_{48+44} (6); however, the data shown in Fig. 1 indicate no significant change in the PfAMA-1_{52}/PfAMA-1_{48+44} band intensity ratio over time at a 1:10 immune serum dilution. Hence, it was concluded that PfAMA-1_{52} was
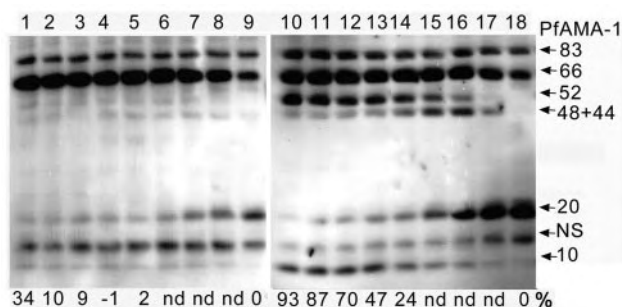
FIG. 2. Effect of inhibitory monoclonal and polyclonal antibodies on AMA-1 processing and merozoite invasion. Schizonts were allowed to rupture in the presence of MAb 4G2dc1 at 1,000, 500, 250, 125, 25, 5, 1, and 0.2 μg/ml (lanes 1 to 8, respectively) or 1,000 μg of noninhibitory MAb 58F8dc1/ml (lane 9). Inhibitory rabbit serum against 3D7 AMA-1 at 20, 10, 5, 2.5, 1.25, 0.25, 0.05, and 0.01% (lanes 10 to 17) and 20% adjuvant control serum (lane 18) was also tested. The invasion inhibition assay was carried out with the first five dilutions of both immune reagents, and percent invasion inhibition values are shown under each lane. nd, the invasion assay was not done.

a terminal product of anomalous secondary processing at a site located C terminal to the normal shedding site of AMA-1 (13). The above observations confirmed the ability of the Western blot assay to separate and detect major AMA-1 processing products.

Comparative effects of inhibitory monoclonal and polyclonal antibodies on AMA-1 processing. Serial dilution of inhibitory MAb 4G2dc1 (Fig. 2, lanes 1 to 8, corresponding to 1,000, 500, 250, 125, 25, 5, 1, and 0.2 μg of MAb/ml) and rabbit serum against recombinant 3D7 AMA-1 (lanes 10 to 17, corresponding to 20, 10, 5, 2.5, 1.25, 0.25, 0.05, and 0.01% immune serum) were tested in a processing inhibition and invasion inhibition assay (Fig. 2). MAb 4G2dc1 significantly inhibited $PfAMA-1_{66}$ processing with little or no $PfAMA-1_{52}$ and $PfAMA-1_{48+44}$ cross-linked to the merozoites. As judged by increased $PfAMA-1_{66}$ and decreased $PfAMA-1_{20}$ band intensities, it was concluded that significant inhibition of secondary processing occurred even at very low, noninhibitory concentrations of the MAb 4G2dc1 (Fig. 2). The serial dilutions of polyclonal serum showed the expected processing-inhibitory profile (6). The $PfAMA-1_{66}$ or the $PfAMA-1_{20}$ band intensity did not correlate with invasion inhibition in the MAb processing assay. $PfAMA-1_{66}$ band intensity in the polyclonal serum processing assay also did not correlate with invasion inhibition. However, in the case of the polyclonal serum, the regression coefficient (R^2) for plots between inhibition of invasion and $PfAMA-1_{20}$ band intensity was 0.342; with $PfAMA-1_{10}$ band intensity it was 0.834; interestingly, a ratio of intensity for $PfAMA-1_{10}$ bands and $PfAMA-1_{20}$ plus $PfAMA-1_{10}$ bands correlated best with invasion inhibition activity, with an R^2 value of 0.942.

Comparative effects of inhibitory polyclonal and monoclonal IgG and their Fab fragments on AMA-1 processing and redistribution. To further discern the mechanism of invasion inhibition, intact IgG and unfractionated Fab fragments of affinity-purified polyclonal antibodies, inhibitory MAb 4G2dc1, and noninhibitory MAb 58F8dc1 were tested in an invasion inhibition assay (Fig. 3A), a processing inhibition assay (Fig. 3B), and a dual immunofluorescence assay (data not shown). It is
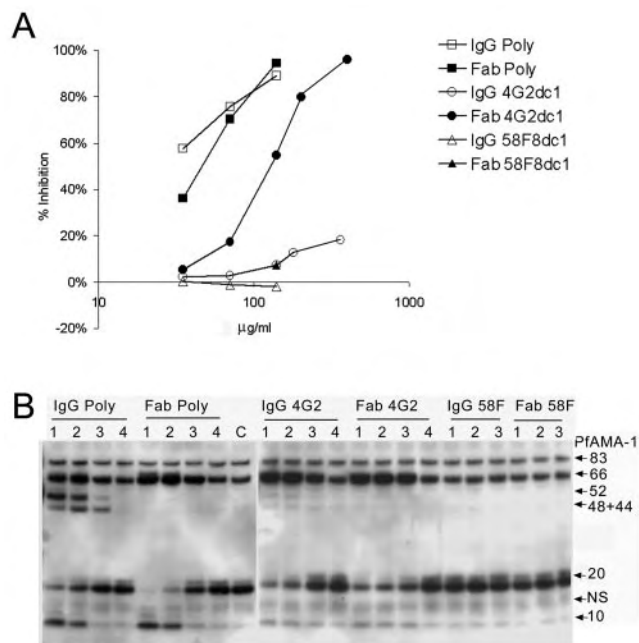

FIG. 3. (A) Plot of concentration (in micrograms per milliliter) of the anti-AMA-1 immune reagents (x axis) and percent invasion inhibition (y axis). (B) Processing inhibition assay with concentrations of 140, 14, 1.4, and 0.14 μg/ml (lanes 1 to 4, respectively) of polyclonal affinity-purified anti-AMA-1 IgG (IgG Poly), unfractionated Fab fragments of polyclonal anti-AMA-1 IgG (Fab Poly), MAb 4G2dc1 (IgG 4G2), unfractionated Fab fragments of MAb 4G2dc1 (Fab 4G2), MAb 58F8dc1 (IgG 58F), unfractionated Fab fragments of 58F8dc1 (Fab 58F), or PBS control (lane C).

notable that at a low concentration (35 μg/ml) affinity-purified polyclonal IgG gave ~60% inhibition, while its Fab fragments showed ~35% inhibition (these data were reconfirmed in two invasion inhibition assays). At a higher concentration, polyclonal intact IgG and its Fab fragments showed equivalent inhibition of invasion (~70% and >80% at 70 and 140 μg/ml, respectively). Intact MAb 4G2dc1 at concentrations of 35, 70, and 140 μg/ml gave <10% inhibition; however, in contrast to the result with polyclonal antibodies, the unfractionated Fab fragments of MAb 4G2dc1 at 140 μg/ml gave 55% inhibition. This difference was more obvious at higher concentrations, where Fab fragments of MAb 4G2dc1 gave 80 and 90% inhibition (200 and 400 μg/ml, respectively), compared to <20% inhibition by the intact MAb (360 μg/ml). Control MAb 58F8dc1 and its unfractionated Fab fragments as well as nonimmune polyclonal rabbit IgG were noninhibitory (data not shown). Figure 3B shows the result of a comparative processing inhibition assay at 140, 14, 1.4, and 0.14 μg/ml (lanes 1 to 4, respectively) of each of the immune reagents tested in the invasion assay. Cross-linking of 52- and 48/44-kDa forms was seen only in the presence of intact polyclonal IgG. All inhibitory immune reagents as well as their Fab fragments inhibited secondary processing of AMA-1, as indicated by the increased $PfAMA-1_{66}$ intensity and a corresponding reduction in $PfAMA-1_{20}$ band intensity compared to the controls. Fab fragments of both polyclonal and monoclonal antibodies were more potent inhibitors of secondary processing than their re-
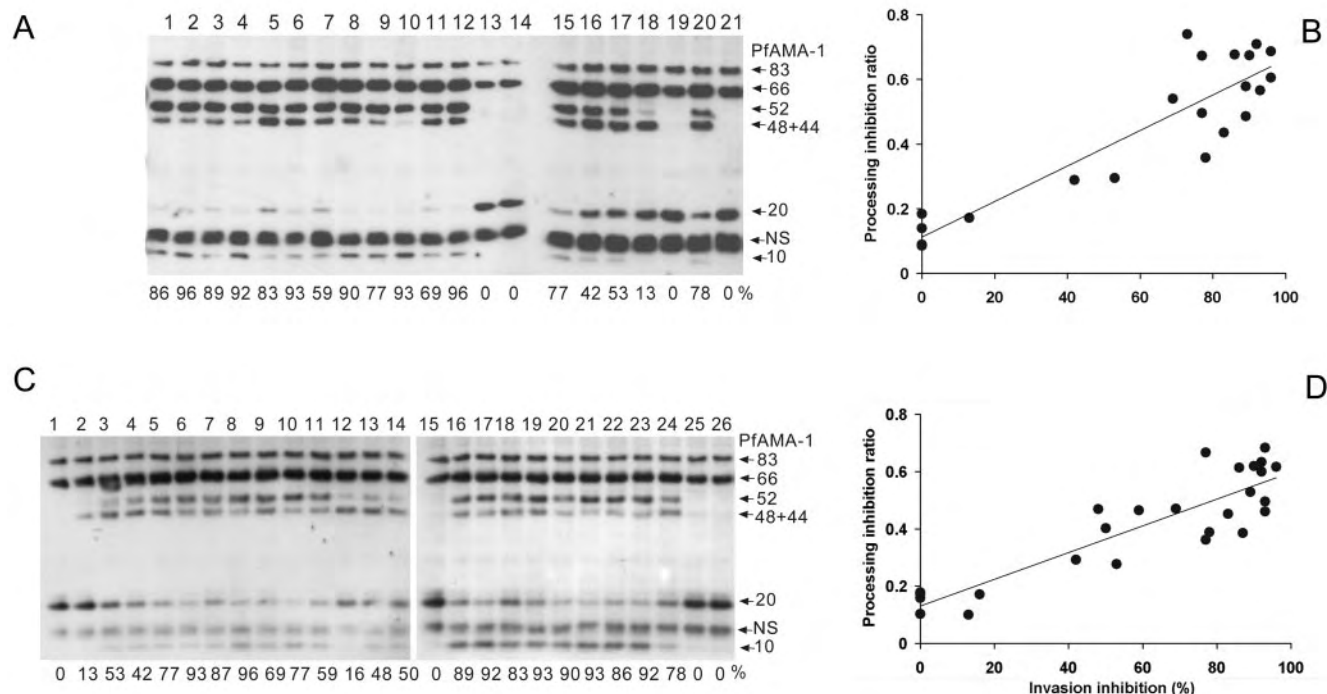
FIG. 4. (A) Processing inhibition assay (at 1:100 dilution) and invasion inhibition assay (at 1:5 serum dilution) with a set of rabbit sera raised against the PfAMA-1 protein from strain 3D7 or strain FVO or a mixture of the two proteins along with adjuvant Montanide ISA720 or ASO2A. IgG preparations from rabbit sera immunized with refolded or reduced and alkylated 3D7 antigen and Montanide ISA 720 were also used. Lanes: 1 to 12, rabbit anti-AMA-1 immune sera; 13, ISA-720 adjuvant control serum; 14, PBS control; 15 to 18, anti-AMA-1 immune sera; 19, preimmune control serum; 20, IgG against refolded AMA-1 protein (3.5 mg/ml); 21, IgG against reduced and alkylated protein (3.5 mg/ml). Percent invasion-inhibitory activities are shown below each lane. (B) Correlation plot between invasion inhibition (*x* axis) and processing inhibition (*y* axis) activity. Processing-inhibitory activity was expressed as the ratio of band intensity of PfAMA-1$_{10}$ versus the intensity of PfAMA-1$_{10}$ plus PfAMA-1$_{20}$. (C) Repeat processing inhibition assay using rabbit sera. Lanes 1, 15, and 26, adjuvant control serum; lanes 2 to 23, immune anti-AMA-1 rabbit serum samples; lane 24, IgG against refolded AMA-1; lane 25, IgG against reduced and alkylated AMA-1. (D) Correlation plot between invasion-inhibitory and processing-inhibitory activities of the serum samples.

spective intact IgG. Intact IgG and Fab fragments of polyclonal antibodies both caused anomalous processing as seen by increased intensity of the PfAMA-1$_{10}$ band; the corresponding PfAMA-1$_{52}$ fragment was only seen in the intact IgG lanes and not in the polyclonal Fab lanes, because Fab fragments failed to cross-link soluble PfAMA-1$_{52}$ to the merozoites. No anomalous processing was observed in the presence of MAb 4G2dc1 or its Fab fragments. Using a dual immunofluorescence assay to localize AMA-1 (circumferential or apical) with respect to MSP-1 (circumferential) on free merozoites (6), it was found that polyclonal intact IgG at 140 μg/ml inhibited circumferential redistribution, while Fab fragments of polyclonal IgG, intact MAb 4G2dc1, or the Fab fragments of MAb 4G2dc1, had no such effect on AMA-1 localization at the same concentration (data not shown). Chymostatin (100 μM) and EDTA (1 mM), both of which are known inhibitors of secondary processing of AMA-1, also did not affect the circumferential redistribution of AMA-1.

Correlation between processing inhibition and invasion inhibition using multiple polyclonal immune sera. A series of individual rabbit sera with invasion-inhibitory activities ranging from 0 to 96% (at 1:5 dilution) were tested in a processing inhibition assay (at 1:10, 1:100, and 1:1,000 dilutions) to determine if processing inhibition activity (defined as the ratio of

intensity of PfAMA-1$_{10}$ bands versus PfAMA-1$_{10}$ plus PfAMA-1$_{20}$ bands) could be used as a predictor for invasion-inhibitory activity of individual sera. The 1:100 dilution was found to be best suited to measure differences in processing inhibition activities of high-titer sera (Fig. 4). Sera used in this analysis included samples from animals immunized with 3D7 AMA-1 protein plus ISA720 adjuvant (two rabbits), FVO AMA-1 plus ISA720 (three rabbits), a mixture of 3D7 and FVO AMA-1 plus ISA720 (eight rabbits), 3D7 AMA-1 plus adjuvant ASO2A (three rabbits), ISA720 adjuvant control (one rabbit), and a preimmune control serum. Rabbit IgG fractions against refolded 3D7 AMA-1 protein (inhibitory) and reduced and alkylated AMA-1 protein (noninhibitory) were also included (3.5 mg/ml, each). As seen in Fig. 4A, anti-AMA-1 rabbit sera showed various degrees of inhibition of PfAMA-1 processing as deduced by the relative intensities of the PfAMA-1$_{20}$ and PfAMA-1$_{10}$ bands. Figure 4B represents the positive correlation obtained between the invasion-inhibitory and processing-inhibitory activities of individual sera (R^2 = 0.809). In a second experiment, sera from 18 rabbits and IgG samples tested in the first experiment were retested along with sera from 6 additional animals immunized with 3D7 AMA-1 plus ISA720 (3 rabbits) or 3D7 AMA-1 plus ASO2A (3 rabbits) (Fig. 4C). As with the previous experiment, a positive
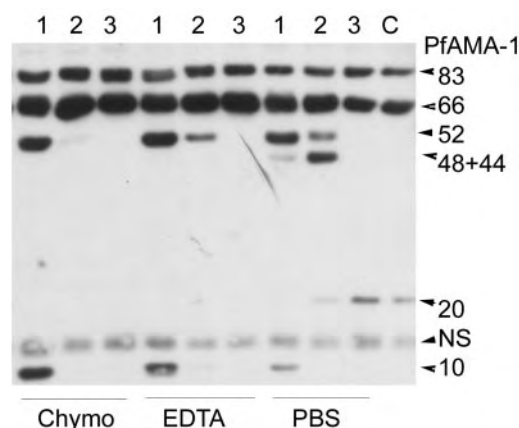
FIG. 5. Effect of immune serum on the processing of AMA-1 in presence of inhibitors of AMA-1 secondary processing. Processing inhibition was assayed in the presence of 100 μM chymostatin (Chymo), 1 mM EDTA, or PBS control and a 1:10 dilution of anti-3D7 AMA-1 serum (lane 1), a 1:2,500 dilution of immune serum (lane 2), or 1:10 dilution of the adjuvant control serum (lane 3). C, no-rabbit-serum control.

correlation of invasion-inhibitory activity with processing-inhibitory activity was observed ($R^2 = 0.787$) (Fig. 4D). The R^2 values between processing-inhibitory activity versus ELISA and between invasion-inhibitory activity versus ELISA plots were much lower (<0.4) in both experiments. Processing-inhibitory activities of antibodies to 3D7 and FVO AMA-1 were proportional to their respective invasion-inhibitory activities against the 3D7 parasite, suggesting that this assay was sensitive to the strain specificity observed in an invasion assay. We did not observe clusters of merozoites in Giemsa-stained preparations of invasion assays performed in the presence of inhibitory polyclonal sera.

Nature of the PfAMA-1$_{52}$ protease. A processing assay was carried out in the presence of a 1:10 dilution of immune serum (lane 1), a 1:2,500 dilution of immune serum (lane 2), or a 1:10 dilution of adjuvant control serum (lane 3) along with known inhibitors of AMA-1 secondary processing (100 μM chymostatin, 1 mM EDTA, or PBS control). This experiment was aimed at confirming the origin of the 52-kDa product of anomalous processing (Fig. 5). The protease activity that resulted in anomalous processing to PfAMA-1$_{52}$ was not inhibited by either chymostatin or EDTA. In fact, both inhibitors promoted anomalous processing in the presence of high concentrations of the immune serum. The extent of anomalous processing (judged by 52- and 10-kDa band intensities) was proportional to the concentration of polyclonal antibodies in the processing assay (note the difference between the 1:10 and 1:2,500 serum dilutions). A broad spectrum of other protease inhibitors were also tested in processing inhibition assays for their ability to inhibit anomalous processing in the presence of a 1:10 dilution of immune serum. Only a serine protease inhibitor, 100 μM tosyl-L-lysine chloromethyl ketone (TLCK), reproducibly inhibited anomalous processing of PfAMA-1$_{66}$ to PfAMA-1$_{52}$. However, TLCK also inhibited schizont rupture (data not shown).

DISCUSSION

Two important malaria merozoite stage antigens, the MSP-1 and AMA-1 of *Plasmodium*, undergo stage-specific processing around the time of invasion (2). Antibodies against MSP-1 that inhibit invasion also inhibit the final step of proteolytic processing, which sheds a 33-kDa MSP-1 fragment from the merozoites, leaving a merozoite-bound 19-kDa fragment (3, 12). We have reported similar inhibition of proteolytic processing of AMA-1 using rabbit polyclonal anti-AMA-1 antibodies (6). Additionally, we found that polyclonal anti-AMA-1 antibodies cross-linked soluble forms of AMA-1 on merozoites, caused anomalous processing, and inhibited the redistribution of native AMA-1 from the apical complex to the merozoite surface. As a step towards developing a functional correlate of AMA-1-based immunity, we examined the evidence for and against the participation of various possible mechanisms of action of anti-AMA-1 antibodies in the inhibition of merozoite invasion into erythrocytes. These are not mutually exclusive, and more than one mechanism may help account for inhibition of invasion.

Antibody-mediated direct steric blocking of biologically functional sites on AMA-1. Merozoites that failed to invade in the presence of both polyclonal and monoclonal antibodies to AMA-1 did not appear to be agglutinated on thin smears, ruling out inhibition of merozoite dispersion as a mechanism of anti-AMA-1 action. Monovalent Fab fragments of two invasion-inhibitory rat MAbs against *Plasmodium knowlesi* AMA-1 are more inhibitory than their intact bivalent IgG forms (25). We show here that the same was true for *P. falciparum*-specific MAb 4G2dc1, indicating a possible role of direct binding of antibodies to biologically functional sites on AMA-1 (Fig. 3). The greater inhibitory effects of Fabs may result from better access of the smaller Fabs to inhibitory epitopes compared to that of their intact IgG forms.

Antibody-mediated cross-linking of 52- and 48/44-kDa forms and inhibition of circumferential redistribution of AMA-1 on merozoites. The only well-characterized cellular process that occurs around the time of invasion and definitively involves AMA-1 is its secondary proteolytic processing and surface redistribution on merozoites (10, 13, 14, 21). Our data suggest that polyclonal antibodies can indeed inhibit both of these cellular processes. Although the roles of either secondary processing or redistribution in the process of invasion remain to be defined, our findings suggest that antibodies to AMA-1 may be inhibiting invasion not just by binding to biologically critical sites on AMA-1, but also by sterically blocking the access of cleavage sites to AMA-1-processing proteases or by cross-linking and blocking the surface redistribution of AMA-1 at the time of invasion. Observations supporting alternative modes of action of anti-AMA-1 antibodies include the following. Intact polyclonal IgG was the most potent inhibitor of invasion among all the monovalent and bivalent reagents tested in this study (Fig. 3); this was also the only immune reagent that showed cross-linking of soluble AMA-1 fragments and inhibition of circumferential redistribution on merozoites. In contrast to the increased inhibition of invasion by monomeric Fab fragments of three MAbs (two against *P. knowlesi* AMA-1 [6] and 4G2dc1 [reported here]), the monomeric Fab fragments of polyclonal anti-*P. knowlesi* and *P. falciparum*

AMA-1 did not possess increased inhibitory activity compared to their respective intact polyclonal IgG. In fact, at a low concentration polyclonal Fab fragments against *P. falciparum* anti-AMA-1 were less inhibitory than their intact IgG forms, similar to the previous findings with *P. knowlesi* (25). It is possible that the inability of polyclonal Fab fragments to cross-link soluble forms of AMA-1 and their inability to inhibit redistribution of AMA-1 on merozoites render them less invasion inhibitory than intact polyclonal IgG. Hence, the increase in the inhibitory activity, due to the steric advantage of Fab fragments, was probably neutralized by a concomitant reduction in invasion-inhibitory effect due to the loss of cross-linking and the loss of inhibition of redistribution activities. We propose that antibody-mediated cross-linking and inhibition of apical restriction may be partly responsible for the enhanced inhibitory activity of polyclonal IgG.

Some reports have assigned an erythrocyte binding role to AMA-1 (7, 27), although the ability of the AMA-1 ectodomain to bind erythrocytes remains controversial (13). Coexpression of *Plasmodium chabaudi* AMA-1 in *P. falciparum* increases the ability of *P. falciparum* merozoites to invade rodent erythrocytes (26), further implicating AMA-1 in erythrocyte recognition. A recent study suggested that the concentration gradient of AMA-1 on free merozoites (highest at the apex and lower towards the basal pole) was either directly involved in reorientation of merozoites on erythrocytes or it may stabilize the merozoite-erythrocyte bond prior to junction formation (probably via the erythrocyte binding proteins [20]). Polyclonal antibody-mediated cross-linking may result in the observed inhibition of circumferential redistribution, thereby preventing the formation of an AMA-1 concentration gradient believed to be involved in reorientation.

Antibody-mediated inhibition of secondary processing. AMA-1 undergoes primary processing from 83 to 66 kDa, and a secondary processing event cleaves the 66-kDa form to 48- and 44-kDa soluble forms and a 20-kDa merozoite-bound fragment (13). Evidence against the importance of inhibition of secondary processing as a mechanism of antibody-mediated inhibition of invasion includes the observation that intact MAb 4G2dc1 causes less inhibition of invasion than polyclonal IgG, although it inhibits secondary processing even more than polyclonal IgG (Fig. 2). Evidence supporting the importance of inhibition of secondary processing includes a direct correlation between antibody-mediated reduction in the intensity of the 20-kDa fragment and the inhibition of invasion in the case of rabbit polyclonal antibodies (Fig. 2 and 4). Fab fragments of both polyclonal and monoclonal antibodies that have better access to AMA-1 also caused enhanced inhibition of secondary processing compared to their corresponding intact IgG (Fig. 3). Protease inhibitors EDTA and chymostatin as well as specific inhibitors designed to block MSP-1 secondary processing inhibited the secondary processing of AMA-1 as well as invasion of merozoites (14). This suggests that antibody-mediated inhibition of secondary processing is associated with inhibition of invasion and may be causally related. The exact biological significance of secondary processing and the resultant shedding of AMA-1 fragments remains speculative. It may be a mechanism by which buried erythrocyte binding sites on AMA-1 are exposed at the time of invasion. Alternatively, secondary pro-

cessing may be an active mechanism to break the AMA-1–erythrocyte contact points, allowing reorientation (20).

Antibody-mediated anomalous secondary processing. In the presence of polyclonal antibodies and their Fab fragments, there was anomalous processing of the 66-kDa fragment to a 52-kDa soluble form and a 10-kDa merozoite-bound form (Fig. 3). Both of these reagents were significantly more potent inhibitors of invasion, even at low concentrations, than MAb 4G2dc1 and its Fab fragments, which strongly inhibit secondary processing but do not induce anomalous processing. Moreover, we found that in the case of polyclonal IgG, including the 10-kDa band in the equation to calculate the processing-inhibitory activity improved its coefficient of regression with inhibition of invasion (compared to considering only the reduction in the 20-kDa band intensity) (Fig. 2 and 4). Hence, anomalous processing of the 66-kDa form may play a role in polyclonal antibody-mediated inhibition of invasion. However, the possibility that the greater potency of polyclonal reagents was due to other factors, including synergistic effects of antibodies of different fine specificities and higher affinities present within the polyclonal sera, cannot be ruled out.

The cleavage site that generates the 52-kDa form is not known. However, the sizes of the products of anomalous processing (52 and 10 kDa) suggest that the cleavage site is either at the end of the stalk sequence connecting the domain III and *trans*-membrane domain or within the *trans*-membrane domain itself (14). MAb 4G2dc1, which did not cause anomalous processing, recognizes an epitope associated with domains I and II of the AMA-1 ectodomain (13, 19). Since the polyclonal sera used in this study contained antibodies to domain III, it is possible that binding of antibodies close to the secondary processing cleavage site is required for anomalous processing to occur. Our data suggest that the inhibitor specificity of the protease responsible for anomalous processing was different from the PfAMA-1 sheddase, which is known to be EDTA and chymostatin sensitive. Although a serine protease inhibitor, TLCK, inhibited the appearance of the products of anomalous processing, we cannot be sure if it was due to the inhibition of the enzyme or due to the inhibitory effects of TLCK on merozoite development and schizont rupture. A TLCK-sensitive and chymostatin-resistant protease was recently shown to be responsible for shedding of PfAMA-1$_{52}$ and PfAMA-1$_{48+44}$ forms from the sporozoites (23). It is possible that this sporozoite-borne AMA-1 sheddase was also active on merozoites and was responsible for anomalous processing. The biological role of anomalous processing is not known. Antibody-induced cleavage at a site further C terminal to the normal shedding site of AMA-1 (producing a 10-kDa instead of a 20-kDa merozoite-associated form) may remove an otherwise functional determinant of invasion, possibly even an erythrocyte binding motif on the 20-kDa fragment. Alternatively, the 10-kDa cytoplasmic domain might dissociate from the membrane and possibly mediate a signaling event that would inhibit invasion or even induce programmed cell death.

With the expected increase in clinical and field trials of malaria vaccine candidates, there will be increasing demand for assays that are functional correlates of protective immunity. The three types of assays described here may be useful in assessing the functional quality as well as the quantity of the AMA-1 antibodies produced in response to vaccination. These

assays measure the ability of antibodies either to inhibit the normal secondary processing of AMA-1 from 66-kDa to 48- and 44-kDa soluble and 20-kDa merozoite-bound fragments, to induce an anomalous processing from 66- to 52-kDa soluble and 10-kDa merozoite-bound fragments, or to cross-link AMA-1 and inhibit the normal circumferential redistribution of AMA-1 on the merozoite. As we have begun to describe and show correlations here, antibodies from vaccinated animals and people could be assayed by the various methods and the results could be correlated with inhibition of merozoite invasion into erythrocytes. Eventually, correlations would also be made with protection against malaria infection. As antibodies become available from people vaccinated with AMA-1, and if they have varying degrees of protection against challenge with malaria, the opportunity will arise to determine the ability of the various assays to correlate with and predict anti-AMA-1-mediated protection.

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