Production of the Subdomains of the *Plasmodium falciparum* Apical Membrane Antigen 1 Ectodomain and Analysis of the Immune Response

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The apical membrane antigen 1 of *Plasmodium falciparum* is one of the leading candidate antigens being developed as a vaccine to prevent malaria. This merozoite transmembrane protein has an ectodomain that can be divided into three subdomains (D I, D II, and D III). We have previously expressed a major portion of this ectodomain and have shown that it can induce antibodies that prevent merozoite invasion into red blood cells in an in vitro growth and invasion assay. To analyze the antibody responses directed against the individual subdomains, we constructed six different genes that express each of the domains separately (D I, D II, or D III) or in combination with another domain (D I+II, D II+III, or D I+III). These proteins were purified and used to immunize rabbits to raise construct-specific antibodies. We demonstrated that D I+II induced a significant amount of the growth-inhibitory antibodies active in the growth and invasion assay.

Several malaria surface proteins expressed during different stages of the parasite’s life cycle have been identified and are being developed as vaccine target antigens. It has been demonstrated that antibodies against membrane proteins of malaria merozoites can, in some cases, block parasite invasion of the erythrocyte (10, 11, 15, 23, 27, 30, 32). One such recombinant vaccine being developed is based on the asexual blood stage integral membrane protein apical membrane antigen 1 (AMA-1) because antibodies to the protein effectively inhibited parasite invasion of erythrocytes in vitro (26, 30, 31, 33). The role of AMA-1 in invasion is further supported by the fact that passive transfer of strain-specific anti-AMA-1 antibodies prevented parasite invasion of red blood cells in the GIA (20). To better understand how antibodies to each of the subdomains of the ectodomain of AMA-1 contribute to the growth-inhibitory effect seen in the GIA, we have expressed subdomain constructs, in single and doublet combinations, in *Escherichia coli*, purified each of the proteins, and raised rabbit antibodies against them. These antibodies allowed us to closely examine the development of AMA-1 as a vaccine for human malaria. Immunization with purified recombinant AMA-1 is protective against the simian malaria parasites *P. knowlesi* (6) and *P. fragile* (2) and the rodent malaria parasites *P. chabaudi* (1) and *P. yoelii* (26). However, the protection was parasite strain specific, suggesting that the protective immune responses were directed toward the polymorphic regions of AMA-1 (5). Protective antibody-mediated immune responses induced against AMA-1 have repeatedly been shown to be directed against conformational epitopes that are dependent on disulfide bond stabilized conformations (1, 2, 5–7, 14, 21, 26). While the amino acid divergence observed among different isolates of *P. falciparum* AMA-1 has been small (~5%), the changes are significant enough, in most cases, to dramatically affect the cross-strain recognition by heterologous protein-induced antibodies (19). The emergence of these differences, at least in D I, has recently been shown to be correlated with symptomatic malaria cases (3).

We recently completed the production and purification of an antimalaria vaccine based on the AMA-1 ectodomain from *P. falciparum* (3D7) (7). Immunization of rabbits with purified protein induced the production of antibodies that significantly (>80%) inhibited parasites in an in vitro growth and invasion assay (GIA). The same level of inhibition in the GIA was observed with whole antibodies and Fab fragments of the antibodies (8). In addition, monoclonal antibodies (MAb) have been produced against the ectodomain that significantly block invasion of red blood cells by merozoites in the GIA (20). To better understand how antibodies to each of the subdomains of the ectodomain of AMA-1 contribute to the growth-inhibitory effect seen in the GIA, we have expressed subdomain constructs, in single and doublet combinations, in *Escherichia coli*, purified each of the proteins, and raised rabbit antibodies against them. These antibodies allowed us to closely examine...
the effect of domain-specific antibodies on the overall inhibition of invasion measured in the GIA.

MATERIALS AND METHODS

Cloning and expression of the recombinant AMA-1 subdomains. Appropriate PCR primers with 5‘ Ncol and 3‘ Notl restriction sites were designed (Bioserve Biotechnologies, Laurel, Md.) for the gene fragments encoding subdomains I, II, III, 1+II, II+III, and I+III (domains as defined by Hodder et al. [13] from the 3D7 isolate of P. falciparum). Gene sequences corresponding to each of these fragments were amplified from a synthetic E. coli codon-optimized AMA-1 ectodomain gene of the 3D7 isolate (encoding amino acids Gly83 to Gly531) by using Taq DNA polymerase and appropriate primers (see Fig. 1). To make D I+III, gene segments dill and dilll were ligated in a separate ligation reaction before TA cloning. The PCR-amplified products were cloned into TA Cloning Vector pCR2.1 Invitrogen, Carlsbad, Calif.). And positive clones were selected by DNA restriction endonuclease analyses and further confirmed by nucleotide sequence analyses. The expression plasmid (7) was restriction digested with Ncol and Notl, and gel-purified gene inserts from the TA cloning vectors were ligated in before transformation into E. coli strain BL21(DE3). In all cases, insertion into the expression vector resulted in His 6 tags on both the amino- and carboxy-terminal ends of the proteins. Bacterial colonies containing expected fragments were picked and analyzed by restriction analysis using pUC19 plasmid DNA as a positive control (Qiagen Inc., Valencia, Calif.). Selected clones were further confirmed by nucleotide sequencing of the plasmid DNA samples. Glycerol (8%) stocks were made for each bacterial clone from an overnight culture and stored at −80°C.

For expression in shake flasks, cells were grown to an optical density at 600 nm of 0.5 and then induced with a final concentration of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested after 2 h of induction by centrifugation at 6,200 × g and 4°C for 30 min. Expression in a 1-liter bioreactor (New Brunswick Scientific, Edison, NJ) used Terrific Broth media (1.2% tryptone, 2.4% yeast extract, 72 mM KH2PO4,2 8m MK H2PO4 [pH 7.2]) containing 15 mM imidazole (pH 8.0). Bound proteins were eluted with 500 mM NaCl, 15 mM imidazole (pH 7.4) followed by 10 cv of 20 mM NaHPO4–50 mM NaCl–10 mM imidazole–0.125% Sarkosyl (pH 7.4) followed by 10 cv of 20 mM NaHPO4–15 mM imidazole (pH 8.0). Bound proteins were eluted with 500 mM imidazole in 20 mM phosphate buffer containing 0.125% Sarkosyl (pH 8.0). Proteins eluted from the Ni-NTA-agarose were rapidly diluted to 30 to 40 mg/ml in a redox-coupled GSH-GSSG solution and allowed to fold overnight as described previously (7). After the refolding period, the protein solution was pH adjusted (to 5.8 for D I and D II, 6.4 for D III, and 6.0 for all double domains) and passed through equilibrated SP Sepharose (Amersham Pharmacia Biotech) (~0.3 ml of resin/g of cell paste). The resin-bound protein was washed with a minimum of 20 cv of 20 mM NaHPO4–1mM EDTA containing 100 mM NaCl (for double-domain constructs) or 250 mM NaCl (for single-domain constructs) at pH 6.0. The bound protein was eluted with 20 mM NaHPO4–1 mM EDTA (pH 7.4) plus 150 mM NaCl (pH 6.0) (for single-domain constructs) or 300 mM NaCl (pH 6.0) (for D I+II) or 400 mM NaCl (pH 6.0) (for D I+III and D II+III).

Purity. Purity was analyzed on precast 4 to 12% Bis-Tris gels (NuPage; Invitrogen, Carlsbad, Calif.) run as recommended by the manufacturer and stained with Coomassie blue. Additional analyses were done using high-performance liquid chromatography (HPLC) using a Waters-510 HPLC pump connected to a Waters-712 WISP auto sampler and controlled by Millen-
present, was about another 20-fold above that. Almost all (~99%) of the parasites harvested from the assays were ring forms or early trophozoites stages, as confirmed by spot checks of Giemsa-stained thin smears. The percent inhibition was calculated from the mean parasitemia of triplicate test and control wells as 100% (test/control). Sera from rabbits immunized with the adjuvant and PBS were used as controls in the GIA. Prebleeds from individual rabbits were tested, and the results were used as controls in the GIA.

To assess the antigen specificity of the antibody-mediated inhibitions, proteins (D I, D II, D III, D I/II, D I/III, AMA-1 ectodomain, or BSA) were added to the IgG preparations (0.5 mg/ml) before the GIA was performed. The final concentration of each test protein in the GIA was 0.5 M.

Statistical analysis. Microsoft Excel was used to calculate the P values for the two-tailed t tests and the correlation coefficients (r²). For the data presented in Fig. 6 and 7, estimates of means, confidence intervals, and hypothesis tests were generated using bootstrap. Each of the available data sets was resampled (bootstrapped) 10,000 times. The observed bootstrap probability estimates reported here are consistent with estimates obtained by assuming that bootstrap means and variances are asymptotically normal. The S language for statistical programming (R v1.8.0) was used to develop the bootstrap estimates. Specific language elements used included the “boot” library and the “Marsaglia-Multicarry” random-number generator.

RESULTS

Expression and purification. All six constructs made from the AMA-1 ectodomain were cloned and expressed in E. coli strain BL-21(DE3). The AMA-1 ectodomain construct, D I+II+III, was made as previously described (7). Figure 1 details the constructs used for individual domains. The biomass yield of a typical 10-liter fermentation was about 400 to 550 g of bacterial paste. All constructs had a His 6 tag, allowing Ni-NTA agarose affinity chromatography as a first step in purification. After refolding, an SP-Sepharose cation-exchange resin was used as a second step of purification, where protein monomers could be separated from multimers using appropriate pH (5.8 to 6.4) and salt concentrations in the wash and elution conditions. This two-step purification yielded >85% pure recombinant domain proteins, as determined from densitometric scans of SDS-PAGE (Fig. 2) and reversed-phase HPLC (data not shown) analyses. All the subdomain fragments of AMA-1 except D I+III resolved into single symmetric peak on a C₄ reversed-phase HPLC column (data not shown). Proteins corresponding to DIII and D I+III had a tendency to precipitate at neutral pH when stored at concentrations above 120 µg/ml. The final yield of purified proteins was 15 to 20 mg/liter of starting culture medium for D I, D II, D I+II, and D I+II+III and 5 to 7 mg/liter for D III and D I+III.

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FIG. 1. Schematic diagram of regions of P. falciparum 3D7 AMA-1 ectodomain expressed for use in this study. Numbers refer to the amino acid residue of the P. falciparum AMA-1 sequence (Gen Bank accession number U65407.1); downward tick marks represent the relative positions of conserved cysteine residues. For D I+III, the construct was a chimera that linked D I residue# 308 to D III residue# 419. Forward and reverse primers, respectively, used for PCR amplification for each of the constructs were as follows: D I, GGAACGGGCGCGAC and CTGCAAGTTTTACGCAAGAC; D II, GCGTAAACCTGCAGAAC and CCGAAAGTGTATTTTTTACTCTC; D III, CTGATTAACACACGCTGAT and CGGCGCTCAGCCTCCTTTAGA; D I+II, GGAACGGCGCGCAG and CGGAAGTGTATTTTTTACTCTCCTC; D II+III, GCGTAAAAACCTGCAGAAC and TGTGGCGGCCGCTTCATCTTTAGA; D I+III, GGAACGGCGCGCAG (-CTGATTAACACACGCTGAT) and CTGCAAGTTTTACGCAAGAC (-GGGCGCTCAGCCTCCTTTAGA), where the sequences in parentheses indicate the end of DI and the beginning of DIII, respectively.

FIG. 2. Coomassie blue-stained SDS-PAGE gel of purified protein from each domain construct, as listed above each panel. The proteins in each panel are (left to right) refolded, reduced, and alkylated. MW, molecular weight.
FIG. 3. Western blot of each purified construct. A 100-ng amount of each purified nonreduced protein was subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose membranes, and reacted with a pool of sera (diluted 1:1,000) from people who lived in a malaria-endemic area of Western Kenya. MW, molecular weight.

Mass and disulfide bond confirmation. The molecular masses of all the constructs were as predicted from the deduced amino acid sequence. The observed average masses of D I, D II, D III, D I+II, D II+III, D I+III, and D I+II+III were 28,895.6 (28,861.3), 18,999.3 (19,004.2), 16,099.4 (16,130.0), 44,113.5 (44,102.4), 29,331.8 (29,340), 41,879.9 (41,882), and 54,656 (54,633) respectively, where the values in parentheses are the predicted masses, and were all within the experimental error limits of measurements of the MALDI-TOF mass spectrometer. The absence of free cysteines was confirmed by an Ellman test. The presence of substantial amounts of free cysteines in the recombinant products would have indicated incorrect folding. All proteins, except the two constructs D III and D I+III, were found to contain less than 0.1 μM free -SH per μmol of protein. The two D III-containing constructs could not be assayed using the Ellman test due to solubility problems at the concentration and pH required for the test. As a second test for disulfide bond-stabilized folding, the proteins were subjected to reduction and alkylation followed by SDS-PAGE analysis. The measurements from the Ellman test show that there were, as predicted from the deduced amino acid sequence of the genes, eight disulfide bonds in D I+II+III, six in D I+III, five each in D I+II and D II+III, three each in D I and D III, and two in D II. All constructs showed significant mobility shifts when subjected to reduction and alkylation (Fig. 2).

Recognition by antibodies. We used several different experimental conditions to determine the extent of recognition of the domains by antisera made against the entire ectodomain (D I+II+III). All six recombinant constructs were recognized on Western blots at the appropriate molecular masses by antibodies in a polyclonal rabbit serum raised against D I+II+III (reference 7 and data not shown) and by a pool of P. falciparum-infected sera collected from western Kenya (Fig. 3). Constructs D I and D I+III were less reactive than the other domains. There was no recognition of these domains in analyses with normal human or rabbit sera at the same concentration (data not shown). MAb 4G2dcI, known to bind to a conformation-dependent epitope within the region encompassing D I+II (16), recognized recombinant D I+II, as shown by Western blotting and ELISA run under nonreduced conditions (data not shown). None of the other constructs were recognized by MAb 4G2dcI either by ELISA or by Western blotting.

Immune analysis of P. falciparum AMA-1 subdomains. In an ELISA format, about 80% of the antibodies made against the ectodomain were directed against D I+II (Fig. 4). In contrast, D I and D II by themselves bound only 6 and 19% of the antibodies, respectively. The other double-domain constructs, D II+III and D I+III, each bound less than 20% of the total antibody.

In a solution competition experiment, the AMA-1 ectodomain (D I+II+III) could deplete 93% of antibodies made against itself while D I+II could deplete 72% of the anti-D I+II+III antibodies. The other double-domain constructs, D II+III and D I+III, could deplete 45 and 51%, respectively. Of the single domains, D I had the highest depletion (~35%), while D II and D III depleted 28 and 26%, respectively.

Recombinant subdomains induce high-titer antibody responses in rabbits. Immunization of rabbits (n = 3) with each of the three double-subdomain fragments induced high-titer antibodies (Table 1). On indirect IFA, all of these antibodies reacted with schizont forms from P. falciparum 3D7 at a dilution of 1:5,000. The reactivity of rabbit anti-double-domain antibodies with P. falciparum AMA-1 was confirmed by immunoblot analyses. Rabbit antibodies obtained by immunization with all three double-domain constructs (D I+II, D II+III, and D I+III) predominantly recognized two bands (Fig. 5) corresponding to the previously identified 83- and 66-kDa full-length and processed forms of AMA1 in P. falciparum (4, 8, 16, 25).

FIG. 4. Percentage of antibodies (y axis) made against the P. falciparum 3D7 AMA-1 ectodomain (D I+II+III) that bind to individual domain constructs. Most of the antibodies generated by D I+II+III were directed to epitopes present in the D I+II region (P < 0.00008). The 100% point is equivalent to the ELISA value of anti-D I+II+III antibody that bound to D I+II+III protein. Error bars represent the variation observed in triplicate wells in two experiments.
Inhibition of *P. falciparum* invasion of erythrocytes. Purified IgG from the pool of double-domain-immunized rabbit sera was used in the GIA. At 1.0 mg/ml, anti-D I+II+III inhibited 77% of parasite development and anti-D I+II resulted in 59% inhibition (Fig. 6). At 0.5 mg/ml, these antibodies resulted in 62 and 42% inhibition, respectively. Antibodies against the other domains or IgG purified antibodies from the preimmune sera did not significantly inhibit invasion in the GIA. To investigate the domain specificity of growth-inhibitory antibodies, the GIA was performed with purified anti-D I+II+III IgG with the addition of recombinant domain proteins to the culture medium (Fig. 7). The addition of DI+II+III protein caused >90% reversal of inhibition in a GIA, and the addition of D I+II and D II+III reversed the growth inhibition of the antisera by 62 and 30%, respectively. There was no significant reversal of growth inhibition in the GIA on addition of D I+III, D I, D II, or D III.

** DISCUSSION **

Several reports suggest that AMA-1 could be an important part of a multicomponent vaccine to combat malaria. AMA-1

![FIG. 5. Western blot of total parasite extract probed with rabbit antisera made against domain constructs D I+II, D II+III or D I+III. *P. falciparum* 3D7 schizonts were extracted with 10% SDS, subjected to SDS-PAGE under nonreducing conditions, and transferred to a nitrocellulose membrane. The blot was treated with postimmune rabbit serum against each of the three double domains, as indicated. Prebleed serum from each rabbit was negative (data not shown).](image)

![FIG. 6. GIA of purified IgG from rabbit sera immunized with each of the multidomain constructs. Solid bars, IgG at 0.5 mg/ml; open bars, IgG at 1.0 mg/ml. Error bars represent the upper and lower confidence intervals of the bootstrap analysis.](image)

![FIG. 7. Reversal of the AMA-1 antibody growth inhibition activity by protein domains. AMA-1 domains were added to cultures of *P. falciparum* 3D7 grown in the presence of 0.5 mg of anti-D I+II+III rabbit IgG per ml. Reversal of inhibition by a specific domain was calculated as the difference of inhibition percentages between cultures containing anti-D I+II+III antibodies and cultures containing antibodies plus 0.5 µmol of that domain per liter. Assays were performed in triplicate, and data in the figure represent bootstrap estimates for 95% confidence intervals on mean reversal, assuming normal distribution. Error bars represent the upper and lower confidence intervals of the bootstrap analysis.](image)
from different *Plasmodium* species are targets of a host immune response to the parasite (18, 26). Knockout of the AMA-1 gene results in inability of the parasites to invade red blood cells, suggesting that it is a pivotal molecule in all *Plasmodium* species (34). Previously we had reported the production of a recombinant AMA-1 ectodomain protein containing all three domains (D I+II+III). Antibodies produced in rabbits to this product recognized the native parasite by indirect IFA and inhibited *P. falciparum* growth by >90% in a one-cycle GIA. To analyze the immune responses to the individual domains and to determine if particular regions of the molecule are better targets for vaccine development efforts, we produced recombinant polypeptides that represented different regions of the AMA-1 ectodomain. While it is impossible to determine the tertiary structure without having crystallographic information, we decided to follow the convention determined by Hodder et al. (13) and divided the ectodomain into three domains based on the loops formed by the cysteine cross-linkages. We have cloned the DNA delineating the domains, either singly or in combination with another domain, to generate six gene constructs (dI, dII, dIII, dI+II, dI+II+III, and dI+III), and expressed them in *E. coli* BL-21(DE3) cells.

Using two chromatographic columns, procedures were developed that yielded recombinant proteins of >85% purity. The final yield of recombinant protein per gram of bacterial biomass was two- to threefold higher for D I+II than for D I+II+III (7), most probably a reflection of the simpler disulfide bond formations in the smaller construct. It has been shown that the disulfide bond-restrained conformation is critical for AMA-1 to induce growth-inhibitory antibodies after vaccination of rabbits (7). MAb 4G2dc1 has been used as a marker to identify a disulfide bond-dependent conformational epitope in AMA-1 (D I+II+III). This MAb bound to D I+II but not to the other single or double domains, indicating that the critical conformational epitope was maintained within D I+II. Our laboratory has produced seven MAbs against D I+II+III, all of which were directed against epitopes that required D II. Similar to 4G2dc1, these antibodies were epitope reduction sensitive (A. Barbosa, unpublished data). Although we do not have data about conformation specific MAbs that recognize D I, D III, or D I+III, recognition of these proteins by immune sera, mobility shifts on SDS-PAGE after reductive alkylation, sharp-and symmetric peaks on RP HPLC analysis, and the absence of free cysteine content in these preparations indicated that they were homogeneous and correctly folded.

Our data indicate that, using our adjuvant with the proteins in rabbit immunizations, D I+II is the most immunogenic region within the *P. falciparum* 3D7 AMA-1 D I+II+III, while D II is the most immunogenic domain among the three single domains. It is evident from direct and competition ELISA that single domains on their own are less immunogenic than the whole ectodomain. This was further confirmed by the reversal of growth inhibition in the GIA. The addition of the D I+II protein to purified IgG made against the entire ectodomain of AMA-1 resulted in significant reversal of the growth-inhibitory capacity of that IgG preparation. The effect was negligible when D I, D II, D III, D II+III, or D I+III was added. The results suggested that single domains, on their own, do not induce significant inhibitory antibodies and that the combination of D I+II plays large role in the total inhibitory activity of anti-D I+II+III antibodies.

To test the potential of domain-specific antibodies to inhibit invasion in the GIA, we immunized rabbits with the purified double-domain proteins D I+II, D II+III, and D I+III. A positive indirect IFA result with late-stage schizonts of *P. falciparum* 3D7 parasites and Western blot recognition of the native AMA-1 from parasite lysate with these antibodies suggested a close similarity of these recombinant preparations to the native parasite antigens and the recombinant vaccine construct containing D I+II+III (7). The ELISA titers were equally high for all three double domains; however, only antibodies from rabbits immunized with the D I+II construct could significantly inhibit parasite growth in the GIA. A low level of inhibition was measured with antiserum to D II+III, and, although D I+III induced high-titer antibodies (titer, 10⁶), these antibodies were unable to inhibit parasite growth in vitro. These data demonstrated that a portion of the region defined within the recombinant D I+II induced a majority of the antibodies that are active in the GIA. Hodder et al. (14) suggested that most inhibitory antibodies were directed against D I because that region contained a majority of the amino acid differences seen among three *P. falciparum* strains (3D7, D10, and HB3) that were inhibited to different degrees in a GIA. Recent studies of D I, the most variable region in AMA-1 (9, 22, 29), suggested that naturally acquired protective immunity targets domain I (3). D I, in the context of D I+III, did not induce any inhibitory antibody, did not significantly inhibit anti-D I+II+III antisera in the competition ELISA, and did not reverse the growth-inhibitory qualities of anti-D I+II+III. While the D I amino acid sequence in the D I+III construct is 11 amino acid residues shorter then the D I domain alone (due to construction restraints encountered during PCR linking of the gene segments), the data still suggest that only in combination with the D II protein does D I express the structured epitopes that potentially can be blocked by antibody to prevent parasite invasion.

Polyclonal antibodies and MAbs against AMA-1 block the invasion of erythrocytes by merozoites. A majority of the epitopes that induce these inhibitory antibodies are conformational and are destroyed on reduction of the disulfide bonds. Due to the problems of disulfide bond formation with proteins expressed in bacteria, the yield of correctly folded full-length ectodomain in this system is low. This study was initiated, in part, to examine the possibility of using smaller AMA-1 constructs, which may be expressed at high levels in bacteria yet still function as effective malaria vaccines. To that end, this study has demonstrated that subdomains of AMA-1 can be expressed at high levels in *E. coli*. In addition, we showed that a polypeptide encompassing a region defined by D I+II can bind most of the antibodies induced on immunization with D I+II+II and that this construct induced potent growth-inhibitory antibodies. However, the overall GIA activity was lower than that observed with antibodies to the D I+II+III construct. Thus, D III may also contain important protective epitopes. Indeed, recently a virosomal formulation containing a synthetic peptide corresponding to D III of AMA-1 has shown growth-inhibitory activity against parasites (24).
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