Alanine Mutagenesis of the Primary Antigenic Escape Residue Cluster, C1, of Apical Membrane Antigen 1

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Antibodies against apical membrane antigen 1 (AMA1) inhibit invasion of Plasmodium merozoites into red cells, and a large number of single nucleotide polymorphisms on AMA1 allow the parasite to escape inhibitory antibodies. The availability of a crystal structure makes it possible to test protein engineering strategies to develop a monovalent broadly reactive vaccine. Previously, we showed that a linear stretch of polymorphic residues (amino acids 187 to 207), localized within the C1 cluster on domain 1, conferred the highest level of escape from inhibitory antibodies, and these were termed antigenic escape residues (AER). Here we test the hypothesis that immunodampening the C1 AER will divert the immune system toward more conserved regions. We substituted seven C1 AER of the FVO strain Plasmodium falciparum AMA1 with alanine residues (ALA). The resulting ALA protein was less immunogenic than the native protein in rabbits. Anti-ALA antibodies contained a higher proportion of cross-reactive domain 2 and domain 3 antibodies and had higher avidity than anti-FVO. No overall enhancement of cross-reactive inhibitory activity was observed when anti-FVO and anti-ALA sera were compared for their ability to inhibit invasion. Alanine mutations at the C1 AER had shifted the immune response toward cross-strain-reactive epitopes that were noninhibitory, refuting the hypothesis but confirming the importance of the C1 cluster as an inhibitory epitope. We further demonstrate that naturally occurring polymorphisms that fall within the C1 cluster can predict escape from cross-strain invasion inhibition, reinforcing the importance of the C1 cluster genotype for antigenic categorization and allelic shift analyses in future phase 2b trials.

The merozoite stage of Plasmodium falciparum is a highly specialized form of the parasite that selectively invades human red blood cells. Although the exact mechanism of invasion is still under investigation, the expression of apical membrane antigen 1 (AMA1) appears to be an absolute requirement for successful invasion (31). Monoclonal and polyclonal antibodies against AMA1 inhibit invasion in vitro, and immunization with recombinant AMA1 protects against live parasite challenge in animal models of malaria (10a, 18, 28).

P. falciparum AMA1 vaccines based on two laboratory strains, 3D7 and FVO, are being studied for efficacy in human trials (23, 27, 30). One of the major concerns in further development of the AMA1 vaccine is that ~10% of its 622 amino acids are polymorphic (2). Strain specificity of vaccine-induced AMA1 antibodies has been observed by enzyme-linked immunosorbent assay (ELISA) and in a functional assay of parasite growth and invasion inhibition (growth inhibition assay [GIA]) (15, 24). Allelic replacement experiments show that sequence polymorphism within AMA1 causes antigenic escape (14), and the extent of escape correlates with sequence distance between the vaccine and target strain (18).

The crystal structure of AMA1 shows that it contains two PAN domains, with loops extending outwards from its central core (1, 22). The loops contain the majority of the polymorphic residues of AMA1 and surround a highly conserved hydrophobic trough. Residues within the trough have been implicated in the binding of AMA1 to AMA1-associated proteins on the merozoite (5). The location of the polymorphic loops surrounding the trough is highly suggestive of its role of providing diversity to a functional region of AMA1, preventing the binding of inhibitory antibodies. Using chimeric proteins to specifically deplete strain-specific antibodies against 3D7 strain AMA1, in a growth inhibition assay (GIA), we showed that certain polymorphic sites conferred escape upon the FVO strain parasite from invasion inhibitory anti-3D7 AMA1 antibodies. These polymorphic residues were termed “antigenic escape residues” (AER). The majority of AER in the 3D7-FVO model mapped to domain 1 (10), and within domain 1, the highest escape per residue was conferred by 7 polymorphic sites located on a linear stretch of sequence between residues 187 and 207. This polymorphic cluster was termed the C1’ cluster (10). It contains the three most polymorphic residues of AMA1, as follows: residue 187 is located on loop 1c, and residues 197 and 200 are located on the adjacent C1-L loop (also termed loop Id).

Given that the polymorphic residues within the C1 cluster are the primary determinants of strain specificity of AMA1, it is likely that protein engineering strategies targeted to C1 AER could be used to modulate the cross-reactivity of AMA1 antibodies. We hypothesized that if the immunogenicity of the C1 AER could be reduced, this might result in an increased antibody response to non-strain-specific cross-reactive epitopes. In this study, seven 3D7-FVO polymorphic differences within the...
C1 cluster were replaced on the FVO AMA1 protein with alanine residues. The 3D7-FVO escape model was then used to study the effect of alanine mutagenesis on the quantity and quality of the induced antibodies. This strategy resulted in a measurable shift in the immune response away from the C1 region but did not enhance the generation of cross-strain-reactive antibodies that were inhibitory to parasite invasion.

MATERIALS AND METHODS

Site-directed mutagenesis, protein expression, and purification. PCR mutagenesis was used to substitute seven polymorphic sites within a synthetic FVO AMA1 gene (based on GenBank accession number CAC05390) for alanine residues, yielding the ALA gene. These 7 residues within the C1 cluster included five polymorphic sites on the C1-L loop and 2 polymorphisms on the adjacent loop Lc (Fig. 1, left). The linear stretch of amino acids that was targeted for mutagenesis included the sequence 187-nPLISPMTLngMRdlFYKnNe-206 (mutated residues are lowercased). Forward and reverse primers corresponding to the sequence 5′-CCG ACC GCC CGG CTG GCT AGC CGG ATG ACC CTG GCC GCC ATG CGC GCT TTT TAT AAA GCC AAC GCA-3′ were used for PCR, in combination with outer primers. Two mutant gene fragments were then amplified using the strain FVO AMA1 gene as a template, and a second round of PCR was conducted to stitch the fragments together. The new ALA-encoding gene was then cloned into a TOPO 2.1 plasmid (Invitrogen, Carlsbad, CA) and sequenced. The ALA gene was further cloned into a modified pET32 plasmid (Retrogen, CA). The four protein chimeras were cloned into a modified pET32 plasmid and sequenced, and the recombinant plasmids were transformed into E. coli Tuner cells (Invitrogen, Carlsbad, CA). Expression of the proteins was conducted in 1-liter shake flasks using Superbroth media containing 30 μg/ml kanamycin. The proteins were purified using in vitro refolding and a two-column purification process essentially as described by Dutta et al. (9).

Antiserum, vaccination, and monoclonal antibodies (MAb). Recombinant FVO or the ALA AMA1 proteins were formulated with Montanide ISA 720 (Seppic Inc., Paris, France) adjuvant (30:70 [vol/vol]). Emulsification was achieved by vigorous vortexing for 10 min. This method has been used routinely in our laboratory to generate high-titer antibodies against AMA1 (9, 10). The formulation was prepared within an hour of vaccination, and antigen integrity was confirmed prior to each vaccination. Each vaccine group consisted of four rabbits, and each rabbit was vaccinated four times with 100 μg of AMA1 administered subcutaneously on days 0, 30, 60, and 90. Rabbits were bled out 2 weeks after the last dose. The immunization site was monitored for adverse local reaction, and the weight of the animals was recorded at 4-week intervals during the study. Pools of antisera against 3D7 strain AMA1 antigen produced during a previous vaccine study (9) were also used. Monoclonal antibody 4G2dc1 was raised against recombinant AMA1 (4) and reacts with a conformational epitope of AMA1 present in all P. falciparum strains. MAbs 1B10, 4E11, and 4E5 were raised against a multiallelic AMA1 vaccine in mice (our unpublished data). MAbs 1B10 and 4E11 recognized the C1 cluster region, whereas 4E5 binds to domain 3 of AMA1 (11). A conformation-specific polyclonal antibody reagent was a 1:5,000 dilution of the anti-FVO AMA1 polyclonal serum (anti-FVO) and preabsorbed with an equal volume of 100 μg/ml reduced-alkylated FVO AMA1 protein. This allowed for depletion of antibodies against linear epitopes, and the resulting conformation-dependent polyclonal antibodies were used for Western blotting (Fig. 1C).

IgG preparation and affinity purification. Serum IgG was purified using a 1-ml protein G resin column per 3 ml serum (GE Healthcare, Piscataway, NJ). Recombinant FVO AMA1 protein (5 mg/ml) was covalently linked to cyanogen bromide (CNBr) Sepharose 4B (GE Healthcare), according to the manufacturer’s instructions. Ten milligrams of purified polyclonal IgG was passed over a
FIG. 2. Monoclonal antibody reactivity. (Top) Dot blot reactivity profile of three novel P. falciparum AMA1 MAbs, 1B10, 4E11, and 4E5, with chimeric AMA1 proteins displaying the P. falciparum 3D7 AMA1 domains D1, D2, and D3 and the hydrophobic trough (HT). (Bottom) Western blot reactivity of the 3D7, FVO, and ALA proteins against the listed MAbs.

1-ml CNBr-AMA1 column, followed by washing with 10 ml phosphate-buffered saline (PBS). Antibodies were eluted using a low-pH IgG elution buffer (Pierce, Rockford, IL) and neutralized by adding 1 M Tris, pH 8.0. IgG was dialyzed against PBS and quantified by measurement at an optical density at 280 nm (OD280). The column flowthrough, wash, and elution were monitored by ELISA for the presence of anti-AMA1 antibodies.

ELISA. Plates were coated with either 3D7 or FVO AMA1 or the reduced-alkylated forms of these antigens (50 ng/well). Plates were blocked with 5% bovine serum albumin (BSA)-PBS for 1 h, sera were serially diluted 4-fold starting from a 1:1,000 dilution, and 75 µl of the dilution was incubated in the wells for 2 h. The plates were washed three times with PBS-0.05% Tween-20. Peroxidase-conjugated anti-rabbit IgG was diluted 1:5,000 in PBS-Tween. Plates were blocked with a 1:4,000 dilution of 3ABT peroxidase substrate (KPL, Gaithersburg, MD). The OD415 of each well was measured after 1 h using a microplate reader ( Molecular Dynamics, Sunnyvale, CA). The antibody titer was calculated as the serum dilution that produced an absorbance of 0.5 optical density units in the ELISA using SoftMax software ( Molecular Dynamics).

Avidity determination by antibody dilution at a single NaSCN concentration. The avidity index was determined by an ELISA method similar to that described by Kneitz et al. (19). Using a multipoint sodium thiocyanate (NaSCN) dilution assay (described below), 2 mM sodium thiocyanate was found to be the minimum discriminating concentration to dissociate low-avidity antigen-antibody binding. We therefore used 2 M NaSCN for the antibody dilution method of avidity measurement. Immunol 2 (Dynex Technologies, Inc., Chantilly, VA) plates were coated with 200 ng antigen in 100 µl PBS per well overnight at 4°C and blocked for 1 h with 2% casein in PBS. The primary antibody was added at a dilution that gave an OD of ~1.4 and at two times this serum concentration in duplicate. After the plates were washed, PBS or a 2 M NaSCN solution was added to sets of duplicate wells and incubated for 15 min. Following a washing, 100 µl of a 1:4,000 diluted anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody was added for 1 h, and plates were washed again. The ELISA was developed by adding 100 µl of ABTS peroxidase substrate for 1 h, and the optical density (OD) was read at 415 nm. The blank OD (no primary antibody blank) was subtracted from the test wells. Optical density was plotted against log serum dilution for both positive and negative NaSCN wells. Serum concentration that resulted in an OD of 1.5 for the negative NaSCN wells was determined using regression analysis. The resulting log serum dilution was substituted into the positive NaSCN regression equation to obtain the OD in the presence of thiocyanate. The avidity index (AI) was described as the percent ratio of OD in the positive NaSCN wells divided by that of OD in the negative NaSCN wells (1.5).

Avidity determination by a multipoint NaSCN dilution at a single serum dilution. For this assay, the ELISA method described by Brujin et al. (6) was used, with minor modifications. Immunol 2 plates were coated with 200 ng antigen in 100 µl PBS per well overnight at 4°C and blocked for 1 h with 2% casein in PBS. The sera were diluted to yield an optical density of ~1.5, and 100 µl was incubated for 1 h. After the plates were washed with PBS-Tween, 100 µl of serial dilutions of NaSCN/well, 0 to 4 M in 0.5 M increments, was incubated for 15 min at room temperature. The remainder of the ELISA was similar to the antibody dilution method described above. After subtracting the blank well OD, the concentration of NaSCN at which 50% of the bound antibodies were eluted (NaSCN that reduced the OD to one-half of the 0 M thiocyanate OD) was calculated using regression analysis and designated the antibody avidity index.

Western blotting. The AMA1 proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed under reducing or nonreducing conditions using a precast 4 to 12% Bis-Tris gel (Invitrogen). The blots were electrochemically transferred to a nitrocellulose membrane and blocked with casein-PBS (Pierce). The blots were incubated with an appropriate dilution of the primary antibody for 1 h and washed with PBS-Tween, followed by 1 h of incubation with a 1:5,000 dilution of an alkaline phosphatase-conjugated secondary antibody (Southern Biotech, Birmingham, AL). The blots were washed and finally developed using nitroblue tetrazolium (NBT)-BCIP (5-bromo-4-chloro-3-indolylphosphate) substrate tablets (Roche, Indianapolis, IN).

GIA and GIAreversal. Sera were thawed at 37°C in a water bath and then heated inactivated at 56°C for 20 min. Ring stages at 0.2% parasitemia and normal red blood cells (RBC) at 2% hematocrit were seeded in a 96-well plate, with a final culture volume of 60 µl. Sera or IgG was tested at a maximum concentration of 16% (vol/vol) in a one-cycle GIA in duplicate or triplicate wells. The plate was sealed in a gas-filled plastic bag and incubated under static conditions for 40 h at 37°C (13). Ring stages of the parasite were stained by adding 12.5 µl of the culture to 500 µl of 0.25% SYBR green dye in PBS at pH 7.4 containing 10 mM EDTA and 10 mM glucose (17). Following 45 min of incubation in a 37°C shaking incubator, parasites were quantified by flow cytometry (488/530 nm) using a BD FACSAccuri (Franklin Lakes, NJ) gated on forward scatter for 40,000 RBC [percent inhibition of invasion = 1 – (percent ring stage parasitemia in test well/percent ring stage parasitemia in immune serum containing control)] For GIA or GIAreversal (FVO or ALA were added at 1:100) GIA at various concentrations. These antigens deplete specific antibodies from the sera, resulting in a reversal of GIA activity. The 3D7 or ALA proteins by themselves do not show any enhancement or inhibition of invasion of the parasite with up to five times the highest antigen concentration tested in the GIA reversal experiment in this study (10).

C1 sequence distance analysis and calculations of escape from invasion inhibition (EII). Cross-strain inhibition experiments were conducted at a 16% serum concentration using anti-FVO AMA1 sera (anti-FVO) from 5 different rabbits (4 immunized during this study and 1 from a previous study [10]) against P. falciparum 3D7, DD2, 7G8, SA154, SA250, CAMP, HB3, and M24 strains. Escape from invasion inhibition (EII) of different strains was calculated for each of the 5 sera [percent EII = 1 – (inhibition of the heterologous strain/inhibition of the homologous FVO strain)]. Using this calculation, the EII for homologous inhibition (FVO target against FVO AMA1 antibodies) always comes to 0%. We also calculated the sequence similarity score of the AMA1 protein for each of the strains used in a cross-strain GIA, using the FVO AMA1 sequence as a reference. Sequence similarity between FVO and each of the 8 target strains was calculated using either all 49 high-frequency polymorphic sites in the AMA1 ectodomain (residues 167 to 544) (7) or the 10 polymorphic differences that occur within these strains at the C1 cluster (residues 187, 189, 190, 196, 197, 200, 201, 204, 206, and 207) (10). A majority of the sites on AMA1 are dimorphic; however, there are 8 positions that accommodate more than 2 alternative amino acids (residues 187, 189, 197, 200, 210, 230, 243, and 244). The similarity score using these “more than dimorphic sites” was also calculated. Sequence alignment and similarity scores were determined using ClustalV software (DNAStar Lasergene 8 software; Madison, WI).

Statistical analysis. Group means were evaluated by analysis of variance (ANOVA) for multiple comparisons to determine if differences existed among the various groupings of data. If the ANOVA P value was <0.05, the differences were further isolated by Tukey’s posttest. Correlation between EII and similarity was established using the Spearman rank correlation. All plots were produced using Prism GraphPad (La Jolla, CA) or Microsoft Excel (Redmond, WA) software.

RESULTS

Expression and folding of the FVO and ALA proteins. The C1 cluster of residues on domain 1 extends from amino acids 187 to 207. There are 7 polymorphic differences between the 3D7 and FVO strains in the C1 cluster. These are residues 187, 190, 196, 197, 200, 204, and 206 (shown in red in Fig. 1, left). Residues 196, 197, 200, 204, and 206 are located on a loop termed C1-L (10), and residues 187 and 190 map to an adjacent loop Ic (1). The ALA protein with alanine residues at all
C1 polymorphic sites and the native FVO AMA1 protein were >95% pure by SDS-PAGE (Fig. 1A) and showed similar reactivity to polyclonal anti-FVO AMA1 (anti-FVO) rabbit immune serum by Western blotting (Fig. 1B). To confirm that the proteins were correctly refolded, both proteins were shown to react similarly to a polyclonal anti-FVO AMA1 serum that had been preadsorbed with reduced-alkylated FVO AMA1 (Fig. 1C). Both proteins lost reactivity to this conformation-dependent antibody reagent under reducing conditions (Fig. 1C, last two lanes). The FVO and the ALA proteins also bound to a MAb, 4G2dc1, which targets a conformation-dependent cross-strain-conserved epitope formed between domains 1 and 2, present only in correctly folded AMA1 proteins (Fig. 1D). These results are consistent with both the FVO and ALA proteins being folded correctly into a biologically relevant conformation.

To establish that the alanine substitutions had altered the surface properties of the ALA protein, binding to three monoclonal antibodies, 1B10, 4E11, and 4E5, was examined. MAbs 1B10 and 4E11 bind to P. berghei AMA1 chimeras that displayed P. falciparum 3D7 AMA1 domain 1 and, within domain 1, the residues surrounding the hydrophobic trough (Fig. 2, top). Western blot analysis showed that MAbs 1B10 and 4E11 bind most efficiently to 3D7 AMA1 but also demonstrated good cross-reactivity with FVO AMA1 (Fig. 2, bottom). Both of the domain 1-specific MAbs 1B10 and 4E11 bound less efficiently to the ALA protein. A control, MAb 4E5, which recognizes a strain-conserved domain 3 epitope, bound efficiently to all three proteins (Fig. 2, bottom). The C1 cluster is located within domain 1 adjacent to the hydrophobic trough (Fig. 1, left). These results are consistent with the ALA protein exhibiting altered surface properties selectively in the mutated C1 region.

Purified ALA and FVO AMA1 proteins were used in a GIA reversal to determine if alanine substitutions in the C1 cluster knocked out epitopes recognized by inhibitory polyclonal antibodies raised against FVO AMA1. Addition of up to 25 μM concentration of AMA1 antigen has no effect on the invasion of FVO parasite (data not shown). Figure 3 shows the mean and 95% confidence intervals of triplicate data collected in two independent experiments. Invasion inhibition was reversed with as little as 0.13 μM FVO and 0.25 μM ALA protein. At low concentrations, the ALA protein was less efficient at reversing inhibition than the FVO AMA1 protein (P was 0.013, 0.0003, and 0.003 in an unpaired, two-tailed t test at 0.13, 0.25, and 0.5 μM antigen concentrations, respectively). This result confirmed that the alanine substitutions altered the surface structure of the C1 cluster region with a reduction in binding to inhibitory anti-FVO antibodies. But interestingly, the reduced ability of the ALA antigen to deplete anti-FVO was largely compensated for by adding a higher concentration of the ALA protein.

Alanine mutagenesis altered the immunogenicity of AMA1. The ALA and FVO AMA1 proteins, formulated with Montanide ISA 720, were used to vaccinate two groups of four rabbits. Sera were obtained 2 weeks after the fourth immunization. Individual serum titers of anti-FVO and the anti-ALA rabbit sera were plotted in Fig. 4, left. Log-transformed titers of ELISA of FVO- and ALA-immunized rabbit sera. (Left) Mean ELISA end-point titers of the individual rabbit sera with the 95% confidence intervals are shown, determined using FVO, ALA, or 3D7 AMA1-coated plates (coat antigen in parentheses on the x axis). (Right) ELISA of anti-FVO and anti-ALA sera titrated against refolded FVO or reduced-alkylated FVO AMA1-coated antigens. The ratios of end-point titers against refolded/reduced protein for individual sera and mean and 95% confidence intervals are plotted.
analyzed by repeated measures of ANOVA revealed an inequality of group means (P = 0.001). The mean titer of the anti-FVO group was higher than that of the anti-ALA group against FVO and 3D7 AMA1-coated plates (Tukey’s multiple comparison; P < 0.05). Although the difference between anti-FVO and anti-ALA group titers did not reach statistical significance against ALA-coated plates, there was a clear trend toward higher antibody titers in the anti-FVO group (Fig. 4, left). Hence, the FVO protein elicited more antibodies that bound to the ALA protein than did the ALA protein itself.

To ensure that the altered immunogenicity resulting from alanine mutagenesis was not due to misfolding of the antigen and higher exposure of linear epitopes, we conducted ELISA of anti-FVO and anti-ALA sera against refolded and reduced-alkylated FVO AMA1-coated plates. As expected, titers for the refolded antigen were higher than those for the unfolded, reduced, and alkylated protein (Fig. 4, right). The ratio of the titers for refolded/reduced protein ranged from 21- to 7-fold for individual rabbits for both anti-FVO and anti-ALA. The mean values of the end-point titer ratio against refolded/reduced antigen for the anti-FVO and anti-ALA group sera were indistinguishable (t test; P = 0.1), confirming that the folded states of the two proteins were similar and that this was not the cause of the altered immunogenicity.

To examine the avidity of the sera, an avidity assay was done using the antibody dilution method (19). The assay was done three times, using 2 M NaSCN as the dissociating agent. Figure 5, left, shows the group mean avidity index and 95% confidence interval for four rabbits per group. Tukey’s multiple comparison test confirmed that the mean avidity index of the anti-ALA group sera was 79% versus 53% against the FVO-coated plate and 72% versus 58% against the ALA-coated plate, respectively (P < 0.05 for both FVO- and ALA coat antigens).

To confirm the above-described observation, the avidity of the rabbit sera was also measured using a NaSCN dilution method (6) at a single antibody dilution (Fig. 5, right). The avidity index, defined by concentration of NaSCN required to reduce the OD to half of the OD at 0 M NaSCN, was higher for the anti-ALA group than for the anti-FVO group. 2.9 M versus 2.2 M against FVO coat antigen and 3.0 M versus 2.2 M against ALA coat antigen, respectively (Tukey’s multiple comparison; P was <0.05 for both FVO and ALA coat antigens). There was no difference in the mean avidity indexes of either the anti-FVO or anti-ALA sera when measured against FVO versus the ALA coat antigen.

Together, these experiments confirm that the FVO and ALA proteins exhibit similar overall properties, with differences confined to the C1 region. Interestingly, the C1 polyalanine substitutions resulted in reduced antibody titers but slightly higher avidity than those of the anti-FVO serum, for both the FVO and ALA proteins bound to the ELISA plate.

Effect of C1 alanine mutagenesis on the cross-reactivity of elicited antibodies. We hypothesized that if the immunogenicity of the AER in the C1 cluster could be reduced, there might be a shift in the immune response to the ALA protein toward its more conserved residues, and thus, it might stimulate more cross-reactive antibodies. To assess the ALA cross-reactive antibody response, total IgG was purified from pools of FVO, 3D7, and ALA AMA1-vaccinated rabbit sera using protein G and titrated against FVO (homologous) and 3D7 (heterologous) AMA1 antigen by ELISA. The 3D7/FVO optical density ratio was plotted against antibody dilution in Fig. 6A. As the antibodies were diluted, the anti-3D7 curve skews toward a 3D7/FVO ratio of >1. In contrast, the anti-FVO curve skews toward higher FVO reactivity (3D7/FVO ratio of <1). In this experiment, an ideal panreactive vaccine that would induce high levels of cross-reactive antibodies would result in an optical density ratio of 1.0 against 3D7- and FVO-coated ELISA wells. However, these data showed that the anti-FVO and anti-ALA curves almost overlapped (paired t test; P was 0.1 for the 3 lowest IgG dilutions shown in Fig. 6A). Hence, no evidence of enhanced cross-reactivity of anti-ALA was observed compared to that of anti-FVO. Indeed, the mean end-point titers of the four FVO vaccinated rabbits were higher than those of the four ALA-vaccinated rabbits against the heterologous 3D7 AMA1 antigen (P < 0.05) (Fig. 4, left). This further
confirmed that the anti-ALA serum did not contain larger amounts of cross-reactive antibodies than anti-FVO.

Full-length antigen ELISA (Fig. 6A) failed to reveal any enhancement of cross-reactivity following alanine mutagenesis. We then tested whether these differences could be more discernible at the domain level. A panel of chimeric proteins displaying the heterologous 3D7 AMA1-specific domains 1, 2, and 3 on a P. berghei AMA1 backbone were used as ELISA coat antigens to quantify domain-specific cross-reactive antibodies. Since the titers of anti-FVO and anti-ALA IgG were different, the individual domain titers had to be normalized by plotting them as percentages of their respective titers against the full-length 3D7 AMA1 protein. Domain-specific ELISA was conducted with IgG purified from pooled sera (Fig. 6B) and using individual rabbit sera (Fig. 6C). Domain ELISA conducted three times on pooled IgG showed that anti-ALA had higher D2 and D3 cross-reactive antibody content and lower D1 specific cross-reactive antibody content than anti-FVO (pairwise t test for three experiments; P < 0.05). Domainwise contents of cross-reactive antibodies were also compared for individual anti-FVO and anti-ALA sera. ANOVA followed by Tukey’s posttest showed no significant difference between anti-FVO and anti-ALA domain specificity (P > 0.05), but there was a trend toward higher mean domain 2 and domain 3 cross-reactive antibody content in the anti-ALA group, which appears to be important, because domain 1 cross-reactivity showed the opposite trend (Fig. 6C).

AMA1, domain 1, elicits fewer cross-reactive antibodies, presumably because this is the most polymorphic region of the molecule, whereas domain 2 and domain 3 elicit more cross-reactive antibodies because these domains are more conserved between strains. Hence, the alanine mutagenesis of strain-specific epitopes on domain 1 did slightly enhance the relative proportion of cross-reactive domain 2 and domain 3 antibodies.

Alanine mutagenesis did not enhance cross-reactive inhibitory activity. To examine the effectiveness of rabbit serum in recognizing AMA1 inhibitory epitopes, anti-ALA and anti-FVO sera were compared in growth inhibition assays (GIAs). Individual rabbit sera from two vaccine groups were tested for inhibition of homologous (FVO) and heterologous (3D7) parasite strains (Fig. 7A). Due to the typical strain specificity of AMA1 antibodies, the mean inhibitory activity of anti-FVO serum was higher against FVO than the 3D7 target strain (Fig. 7A, top, thick lines). The GIA activity of anti-FVO against the FVO target was significantly higher than that of anti-ALA only at the two lowest serum concentrations (4% and 2% [vol/vol]). At higher serum concentrations and against the 3D7 target parasite, the mean anti-FVO and anti-ALA group inhibitions were not statistically different (multiple comparisons done using ANOVA, followed by Tukey’s posttest; P > 0.05). Most importantly, anti-ALA serum showed no improvement in the

FIG. 6. Cross-reactivity and domain specificity of FVO and ALA antibodies. (A) ELISA with 3D7 and FVO AMA1-coated plates using serial dilutions of pooled purified anti-FVO and anti-ALA IgG preparations (starting at 29 mg/ml IgG). The ratio of OD415 against 3D7 AMA1 to that of the FVO AMA1-coated wells was plotted against IgG dilution (x axis). (B) A domain end-point ELISA against 3D7 D1, D2, and D3 chimeras was conducted using pooled anti-FVO and anti-ALA IgG (rabbit group FVO or ALA in parentheses). The end-point titer against individual domains was expressed as a percentage of the titer against the full-length 3D7 AMA1. The mean value from 3 independent experiments for the IgG ELISA was plotted, along with the P
level of cross-strain inhibition of the 3D7 parasite (Fig. 7A, bottom).

In the above-described experiment (Fig. 7A) using whole serum, the inhibition did not reach a maximum at the 16% serum concentration (highest concentration tested). To overcome this, a GIA was also conducted using protein G-purified and concentrated IgG. The anti-FVO IgG showed a higher level of inhibition of the homologous FVO parasite, reaching saturating inhibition of 80% at 3 mg/ml, while anti-ALA IgG showed the same inhibition at twice the IgG concentration (Fig. 7B, top and bottom, solid lines). This confirmed that anti-FVO IgG was a more potent inhibitor of invasion. The inhibition of the heterologous 3D7 strain was again comparable between anti-ALA and anti-FVO (Fig. 7B, top and bottom, solid lines), confirming that alanine mutagenesis did not enhance cross-reactive inhibition of the heterologous parasite strain. In order to determine if there was a difference in cross-reactivity against strains other than 3D7 and FVO, a GIA was also carried out using the protein G-purified IgG against parasite strains 7G8 and CAMP (Fig. 7C). No differences in the cross-strain inhibition pattern were observed for anti-FVO and anti-ALA IgG. This indicated that, although there was a trend toward detectable domain-specific enhancement of cross-reactive response to domain 2 and domain 3 (Fig. 6B and C), these cross-reactive antibodies did not target invasion inhibition-susceptible epitopes on AMA1.

Reduced inhibitory activity of AMA1 affinity-purified anti-ALA IgG. To compare the AMA1-specific inhibitory activity levels, the protein G-purified IgG was further affinity purified by binding and elution from an FVO AMA1 column. The flowthrough and the elutions of anti-FVO and anti-ALA were titrated by ELISA to confirm the almost-complete extraction of anti-AMA1 IgG by the FVO AMA1 affinity column (data not shown). The eluted anti-AMA1-specific IgG was then quantified and used in a GIA at various concentrations. The 50% effective dose (ED$_{50}$) of IgG that gave 50% inhibition was calculated using a four-parameter curve. Figure 8 shows the mean and 95% confidence intervals of the ED$_{50}$s from three separate experiments. The mean ED$_{50}$ of anti-FVO against FVO parasites was 0.34 mg/ml, whereas a significantly higher concentration of anti-ALA, 0.56 mg/ml, was necessary to achieve a similar level of inhibition (ANOVA, followed by Tukey’s multiple comparison; $P < 0.05$). This result was expected, because a critical inhibition-susceptible epitope had been altered in the ALA protein. The mean ED$_{50}$ of anti-FVO against 3D7 parasites was 0.75 mg/ml. As expected, a higher level of antibodies is necessary for cross-strain inhibition. But, disappointingly, the ED$_{50}$ of anti-ALA against 3D7 parasites of 1.26 mg/ml was significantly higher than the ED$_{50}$ of anti-FVO ($P < 0.05$). This result refutes our hypothesis that a mutation of a polymorphic region of AMA1 will lead to enhanced cross-strain inhibition. The C1 polyalanine mutation
resulted in altered recognition of conserved regions of AMA1 (Fig. 6B and C), but this result indicates that these were not inhibition-susceptible epitopes. These data presented in Fig. 8 emphasize the importance of the C1 epitope in eliciting invasion inhibitory antibodies.

**C1 sequence similarity can be used to predict parasite EII.** The alanine mutagenesis data showed that C1 AER play a critical role in the induction of inhibitory antibodies. To determine if the genotype of the C1 cluster residues could predict strain-specific escape from invasion inhibition in a GIA, sera from 5 FVO AMA1-immunized rabbits were tested for inhibition of 8 *P. falciparum* strains, 3D7, DD2, 7G8, SA154, SA250, CAMP, HB3, and M24, in a GIA. The percent inhibition against the homologous FVO strain was used to normalize invasion inhibition of different serum samples and to calculate escape from invasion inhibition (EII) against FVO AMA1 antibodies. The percent amino acid similarity score between the vaccine strain (FVO) and the target strain AMA1 protein was plotted against the percent EII (Fig. 9). When the sequence similarity was calculated using 49 high-frequency polymorphic residues within the AMA1 ectodomain (7), the Spearman rank correlation analysis resulted in a rho factor of −0.35 and *P* value of 0.07 (Fig. 9, left). If the polymorphic residues that were exclusively dimorphic were excluded and correlation was plotted using the 8 more than dimorphic sites, the rho factor improved to −0.48 and the *P* value was 0.01, indicating statistically significant correlation. However, when the Spearman rank analysis was restricted to 10 polymorphic differences in the C1 cluster (residue 187 to 207), the rho factor was better still at −0.746 and the *P* value was $1.2 \times 10^{-3}$, demonstrating a more significant correlation (Fig. 9, right). Note, although there are 7 FVO-3D7 polymorphic differences in C1, there are 10 C1 polymorphic differences when comparing all the strains. These data indicate that the sequence distance of only 10 C1 cluster polymorphisms was sufficient to predict susceptibility of a target parasite to an AMA1 vaccine and is consistent with this region of AMA1 playing a dominant role in terms of immunogenicity and susceptibility to inhibition.

**DISCUSSION**

Epitope-silencing strategies described in the literature remain largely empirical. These include epitope masking by glycosylation, epitope deletion, and alanine mutagenesis. These strategies have most often been utilized to enhance the cross-reactivity of HIV vaccine candidates, where antigenic escape is a major hurdle to vaccine development. One of the immunodominant epitopes of HIV is the third hypervariable loop, V3, of the virus receptor protein gp120. Introduction of an N-linked glycosylation site at V3 diverted the immune response of GP120 to the V1 loop (11). Interestingly, in the absence of V3 immunodampening, V1 is a nonneutralizing epitope; however, following V3 glycosylation, the V1 epitopes become immunodominant and neutralizing (11). Shortening the V3 stem escape from invasion inhibition (EII) against FVO AMA1 antibodies.
was also found to reduce its immunogenicity and, when combined with a V1/V2 deletion, elicited more broadly neutralizing antibodies (32). Polyalanine mutagenesis combined with N-linked glycosylation was used to generate a variant of gp120 that was selectively recognized by the broadly inhibitory b12 MAb, but this gp120 variant failed to elicit broadly neutralizing antibodies (26). In general, glycosylation, deletion, or mutagenesis of epitopes can be utilized to modulate the immune response, but effects on immunogenicity are often unpredictable.

Malaria antigens are naturally nonglycosylated, and alanine mutagenesis was adopted as an immunodampening strategy. Alanine is a unique amino acid in that it has a low probability of being part of a B-cell epitope, and yet, it has low hydrophobicity such that it is likely to be well tolerated on a protein surface (12). A triple alanine mutation successfully immune silenced the A2 region of recombinant human factor VIII, resulting in reduced antibody inhibition and more effective clotting in hemophilia A mice (21). The basis for the reduced immunogenicity imparted by alanine residues is not clear but may result from reduced epitope flexibility due to an absence of alternative side chain conformations. Reduced flexibility will decrease the chance of antibody binding as a result of diminished induced-fit possibilities.

In this study, 7 surface residues of FVO AMA1 were mutated to alanine in the C1 cluster. These mutations altered the binding of monoclonal antibodies 1B10 and 4E11, which bind to the hydrophobic trough region of AMA1, but did not significantly alter the overall folding of the molecule. This was confirmed by a high level of reactivity of the ALA protein to a conformation-sensitive MAb, 4G2dc1, and a polyclonal anti-FVO rabbit serum that was depleted of antibodies against linear epitopes. Hence, the immunological differences between the FVO and ALA proteins were most likely due to alteration of the C1 surface. AMA1 peptides have been shown to induce T-cell proliferation in malaria-infected humans (20). Although none of these T-cell epitope maps to the C1 cluster, the possibility that the C1 polyalanine substitutions altered a T-cell epitope on AMA1 cannot be ruled out.

In rabbits, the ALA protein consistently induced lower antibody titers than FVO, suggesting that epitopes within the C1 region were successfully immunodamped. There was also a trend toward a higher proportion of domain 2- and domain 3-specific cross-reactive antibodies in the anti-ALA sera than in anti-FVO. This observation suggests that immunodampening had shifted the overall immune response in favor of conserved epitopes on domains 2 and 3, as we had originally aimed. Anti-ALA, however, showed no improvement in the cross-strain inhibition of heterologous strains and, in fact, was less inhibitory than anti-FVO serum, as determined by ED₅₀ measurements. Hence, despite the shift in favor of novel conserved epitopes, there was a corresponding decrease in the immunogenicity of inhibition-susceptible epitopes in favor of noninhibitory epitopes, confirming that the C1 region was an important component of the inhibitory response against AMA1.

Interestingly, despite its lower titer, the overall avidity of anti-ALA serum was higher than that of anti-FVO serum. This observation could be explained by the shift in the immune response toward the domain 2 and 3 epitopes (Fig. 6B and C). Presumably, antibodies elicited by the C1 cluster polymorphic residues of the FVO protein were of low avidity, whereas the alternative domain 2 and 3 epitopes on the ALA antigen elicited higher-avidity antibodies. We speculate that the flexibility of loops present in the C1 region leads to the immunodominance of its polymorphic residues, due to increased induced-fit possibilities. However, due to unfavorable conformational entropy, the resulting loop-recognizing antibodies are of low avidity. Polymorphic loops may therefore mediate immune evasion by reducing the avidity of antibody binding close to a functional region—the hydrophobic trough of AMA1 (Fig. 2) (1).

The majority of the work highlighting the C1 AER was originally generated by in vitro mapping of rabbit polyclonal antibodies (10). An inhibitory monoclonal antibody 1F9 (3) was also mapped to the C1 region, and this epitope was shown to be targeted by the anti-AMA1 antibodies elicited in humans (3). More recently, sequence analysis of longitudinally collected parasite isolates from an area of disease endemicity indicated that polymorphisms in the C1 AER were associated with the development of clinical malaria symptoms during serially acquired infections (29). We confirm here that an inverse correlation exists between the C1 sequence similarity and the ability of a parasite strain to escape invasion inhibition in cross-strain growth inhibition assays. The genotype of the C1 AER may therefore be useful to classify AMA1 into allelic types for rational development of a polyvalent vaccine and for allelic shift analyses in the phase 2b trials of mono- or biallelic AMA1 vaccines (25).

There are 7 amino acid differences between 3D7 and FVO strain AMA1 that lie within the C1 cluster (positions 187, 190, 196, 197, 200, 204, and 206); at these sites, FVO AMA1 possesses amino acids L, D, E, H, F, K, and Y, whereas the 3D7 AMA1 amino acids are L, N, G, D, F, E, and Y. Amino acids at position 187 (L), 200 (F), and 206 (Y) are conserved in both FVO and 3D7 AMA1. Therefore, even though the C1 cluster is the most polymorphic region of AMA1, cross-strain epitopes are likely to exist in this region, accounting for C1-dependent differences in cross-strain inhibition of 3D7 parasites (Fig. 8). The radical polyanaline substitution within the C1 cluster of the FVO protein not only disrupted the critical strain-specific epitopes but also may have disrupted these cross-strain inhibitory epitopes. The use of less radical amino acid substitutions (residues other than alanine) might be a more viable strategy to preserve the cross-strain inhibitory epitopes while immunodampening the strain-specific epitopes.

Monoclonal antibody 4G2dc1 recognizes a cross-strain inhibitory epitope on AMA1 (4). On a molar basis, 4G2dc1 shows significantly less cross-strain inhibition compared to that shown by affinity-purified polyclonal antibodies (8). We speculate that the reason for high inhibitory activity of polyclonal antibodies against AMA1 is that efficient parasite inhibition requires synergy between antibodies binding to nonoverlapping epitopes on AMA1. This is consistent with a surprising result presented in Fig. 3. At high levels, both the FVO and ALA proteins were almost equally effective at competing for inhibitory antibodies, suggesting that C1-binding antibodies, by themselves, are not efficient mediators of invasion inhibition. Although immune responses in the rabbit model may not reflect human immune responses to AMA1, our observations using rabbit antibodies emphasize the need for comprehensive
mapping and identification of other inhibition-susceptible regions of AMA1. Epitope display vaccine technologies that favorably present cross-strain inhibitory epitopes to the immune system may be essential for AMA1-based vaccines to impart protection in humans.

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