Passive Immunization with a Multicomponent Vaccine against Conserved Domains of Apical Membrane Antigen 1 and 235-Kilodalton Rhoptry Proteins Protects Mice against *Plasmodium yoelii* Blood-Stage Challenge Infection

David L. Narum,1* Solabomi A. Ogun,2 Adrian H. Batchelor,3 and Anthony A. Holder2

*Corresponding author. Mailing address: Malaria Vaccine Development Branch/NIH, 5640 Fishers Lane, Twinbrook I, Rockville, MD 20852. Phone: (301) 435-2185. Fax: (301) 480-1962. E-mail: dnarum@niaid.nih.gov.

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During malaria parasite invasion of red blood cells, merozoite proteins bind receptors on the surface of the erythrocyte. Two candidate *Plasmodium yoelii* adhesion proteins are apical membrane antigen 1 (AMA1) and the 235-kDa rhoptry proteins (P235). Previously, we have demonstrated that passive immunization with monoclonal antibodies (MAbs) 45B1 and 25.77 against AMA1 and P235, respectively, protects against a lethal challenge infection with *P. yoelii* YM. We show that MAb 45B1 recognizes an epitope located on a conserved surface of PyAMA1, as determined by phage display and analysis of the three-dimensional structure of AMA1, in a region similar to that bound by the *P. falciparum* AMA1-specific inhibitory antibody 4G2. The epitope recognized by 25.77 could not be assigned. We report here that MAbs 45B1 and 25.77 also protect against challenge with the nonlethal parasite line 17X, in which PyAMA1 has a significantly different amino acid sequence from that in YM. When administered together, the two MAbs acted at least additively in providing protection against challenge with the virulent YM parasite. These results support the concept of developing a multicomponent blood-stage vaccine and the inclusion of polymorphic targets such as AMA1, which these results suggest contain conserved domains recognized by inhibitory antibodies.

Passive immunization with purified immunoglobulin derived from adults living in areas holoendemic for malaria protects against *Plasmodium falciparum*, the causative agent of the most severe form of human malaria (7, 44). Human studies such as these have provided the framework for the development of a malaria vaccine to induce protective antibodies against the erythrocytic stage of the parasite’s life cycle. Several parasite proteins have been identified as targets of these protective antibodies, and there is considerable interest in developing a vaccine that induces a response to more than a single protein, and there is considerable interest in developing a vaccine that induces a response to more than a single protein, although the benefit of targeting multiple proteins has not been well established experimentally (23). A vaccine study in an *Aotus* monkey model with a combination of two recombinant blood-stage *P. falciparum* proteins, the 42-kDa C-terminal fragment of merozoite surface protein 1 (MSP142) and apical membrane antigen 1 (AMA1) failed to detect any additive or synergistic response (48).

A number of malaria models have been used to define the immune mechanisms responsible for protection in humans. Rodent malaria parasites such as *Plasmodium yoelii* in laboratory mice have been used to study the role of antibody responses to parasite proteins. This approach is of particular value for orthologues of *P. falciparum* proteins such as MSP1 (27) and AMA1 (38, 53, 57). Active and passive immunization studies with PyMSP1 have shown that antibodies directed against the C-terminal region protect against erythrocytic development and multiplication of the parasite (17, 27, 46), even in knockout mice devoid of a functional Fc receptor (43).

AMA1 has been found in all *Plasmodium* species studied and is a merozoite protein that is a target of antibodies that neutralize invasion of erythrocytes (30, 52). PfAMA1 is a promising malaria vaccine candidate (28) for which the crystal structure has recently been reported (2). Rhesus monkeys immunized with native *P. knowlesi* AMA1 (11) or mice immunized with recombinant *P. chabaudi* AMA1 (1) were protected against blood-stage challenge infection. Rat monoclonal antibodies (MAbs) and their Fab fragments against native *P. knowlesi* AMA1 inhibited merozoite invasion in vitro (10, 52), a finding consistent with the idea that AMA1 is a parasite ligand involved in merozoite invasion of erythrocytes. Although the specific function of AMA1 during merozoite invasion is unknown, PyAMA1 has been identified as an erythrocyte binding protein, and MAb 45B1 against PyAMA1 blocks this ligand-receptor interaction (12). Native PyAMA1 induces a protective response, and passive immunization with MAb 45B1 protects against a lethal blood-stage infection with the YM line of *P. yoelii* (32).

Another target of antibodies that protect against the virulent YM parasite is the family of high-molecular-mass rhoptry proteins (PyP235) (13, 18). PyP235 is coded by a multigene family (4, 31), and at least one of the PyP235 proteins binds to the surface of mouse erythrocytes (35, 36), suggesting that they are involved in erythrocyte recognition and invasion. PyP235 proteins are members of a superfamily that includes reticulocyte binding proteins of *Plasmodium vivax* (14) and proteins re-
cently identified in *P. falciparum* (41, 50, 55). Immunization with PyP235, or passive immunization with two MAbs (25.77 and 25.37) directed against PyP235, protects BALB/c mice against challenge with the virulent *P. yoelii* YM line (13, 18), restricting the parasite to reticulocytes. PyP235 binds preferentially to mature erythrocytes, and the binding is inhibited by specific antibodies, suggesting a role in the invasion of mature erythrocytes but not reticulocytes (37).

Given that passive immunization of humans and mice with antibody is protective against malaria blood-stage infections, the rodent model provides a useful tool to answer additional questions. These questions include protection against diverse parasites that contain antigenic polymorphisms within a target protein and how to model the effects of targeting multiple proteins in a multicomponent blood-stage malaria vaccine. To study the effects of parasite diversity on protection mediated by passive immunization and to model the additional efficacy of a multicomponent blood-stage malaria vaccine, we chose to investigate two *P. yoelii* blood-stage proteins: PyAMA1 and PyP235.

We screened a constrained phage-display library to identify the epitopes recognized by MAbs 45B1 and 25.77 within PyAMA1 and PyP235, respectively. The epitope recognized by MAb 45B1 was defined, and it was shown that a peptide mimotope for MAb 45B1 competes with native PyAMA1 for antibody binding. The location of the mimotope was mapped to a conserved region on the surface of PyAMA1 using the *P. falciparum* AMA1 crystal structure (2). The distribution of the 45B1 epitope in various *Plasmodium* species and subspecies of *P. yoelii* was examined. The epitope recognized by MAb 25.77 could not be unambiguously identified, but it is known to be conserved (31). These two MAbs were shown to attenuate the normal progression of infection of the nonlethal *P. yoelii* 17X line that expresses diverse AMA1 and P235 proteins (21, 40). Most importantly, it was shown that passive immunization with a combination of the two MAbs provided an additive effect in protection against the lethal blood-stage infection of the YM line, providing evidence to support the development of a multiple component blood-stage vaccine.

**MATERIALS AND METHODS**

**Parasites.** Rodent parasites including cloned lines of *P. yoelii yoelii* 17X (accession no. U45971) and YM (accession no. U45970) (58) were obtained from David Walliker, University of Edinburgh, and grown in BALB/c mice. Slides were prepared with thin smears of blood parasitized with *P. falciparum* lines (FCB-1 and 3D7 (accession no. AAA63444 and AF298218), *P. y. yoelii* isolates (33X, 2CL, 2BR, 3AE, and 1AR); *P. yoelii* killicki and *P. yoelii* nigeriensis (3); and *P. berghei* (accession no. CAA76546), and a region of marked homology was identified within domain 1 of the sequence in phage clones 1 and 2 flanked by cysteine (Gln) was present. Using a high-titer phage preparation (Lys

**Antibodies.** The protective mouse MAb 45B1 recognizes *P. yoelii* YM AMA1 and MAb 48F8 recognizes a 140-kDa *P. yoelii* apical merozoite protein (32). The protective mouse MAb 25.77 recognizes a member of the P35 family (18). Rat MAB 2SG2 recognizes the C terminus of AMA1 within all *Plasmodium* species tested (33). Immunoglobulin G was purified by protein G affinity chromatography as previously described (33). The immunoglobulin G (IgG) concentrations were determined by measuring the absorbance at 280 nm and with an extinction coefficient of 1.4.

**Screening of the phage display library.** The Ph.D.-C7C, a disulfide constrained 7-mer phage display library (New England BioLabs, Beverly, MA) was screened as recommended by the manufacturer. After a third round of amplification for binding to MAB 45B1 or a fifth round of amplification for binding to MAB 25.77, individual phage were isolated, and the peptide sequences they displayed were deduced after DNA sequencing.

**Peptide synthesis.** The peptide PEP45B1 (CSNSDKPKCGGGS) and a control peptide (CGDNSDRPGGGGSS) were synthesized, cyclized by oxidation at high dilution, and purified to at least 95% by high-performance liquid chromatography (Infiniti Biotech Research and Resource, Aston, PA).

**Immunofluorescence assay.** For immunofluorescence assay (IFA), thin films of blood were prepared as previously described (33) and stored desiccated at −70°C. Thin films were made from blood with a parasitemia ranging from 2 to 20% and were incubated with primary antibody in buffer at 10 μg ml−1 for 30 min. The slides were washed and then incubated for 30 min with goat anti-mouse IgG conjugated to fluorescein (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). After further washing, the slides were examined by fluorescence microscopy.

**Competitive ELISA.** High protein-binding, 96-well, flat-bottom microtiter enzyme-linked immunosorbent assay (ELISA) plates (Nunc Microwell Microplates; Fisher Scientific, Pittsburgh, PA) were coated with AMA1 purified from a lysate of *P. yoelii* YM-infected erythrocytes (at approximately 25 ng ml−1 in phosphate-buffered saline [PBS]–sodium azide buffer) and incubated overnight at 4°C. Peptide PEP45B1 was serially diluted in antibody buffer (PBS [pH 8.0] containing 0.5% bovine serum albumin) containing MAB 45B1 IgG at 10 μg ml−1 and incubated for 1 h at 37°C. After washing, goat anti-mouse IgG conjugated with alkaline phosphatase (Promega, Madison, WI) at a dilution of 1:7,500 was added in a volume of 50 μl per well, followed by incubation for 1 h at 37°C. Blue Phos phosphatase substrate (Kirkegaard and Perry) was added to each well, and the absorbance at 635 nm measured. The observed absorbance measurements were low due to limited amounts of purified PyAMA1 for plate antigen.

**Passive immunization and parasite challenge.** Passive immunization studies were conducted with groups of three or six BALB/c mice 6 to 8 weeks of age by intraperitoneal administration of IgG in PBS on days −1, 0, and +1 relative to challenge, as previously described (32). On day 0 mice were inoculated intravenously with 5,000 parasitized red blood cells. Thin smears of tail blood stained with Giemsa reagent were examined daily after day 3 postchallenge, and parasitemias were scored on alternate days. Mice were sacrificed if the parasitemia exceeded 70% or if they showed defined clinical signs.

**Statistical analysis.** Differences among mean parasitemias were analyzed with one-way analysis of variance with Tukey’s HSD test used for post-hoc analysis (SPSS, version 11; SPSS, Inc., Chicago, IL).

**RESULTS**

**Isolation of phage and identification of a mimotope for the PyAMA1-specific MAB 45B1.** To try to identify the corresponding epitopes, a cyclic (disulfide bond-constrained) random 7-mer peptide phage-display library was screened with the AMA1-specific MAB 45B1 and the P235-specific MAB 25.77, and phages binding to the antibodies were isolated. The deduced seven-residue amino acid sequences derived from the phage clones selected by panning against MAB 45B1 (Fig. 1A) were aligned with the *P. yoelii* YM AMA1 protein sequence, and a region of marked homology was identified within domain II (residues 290 to 296 in the PyAMA1 sequence). Examination of phage clones 1, 2, and 5 showed that five of seven amino acids were identical to the corresponding AMA1 sequence, and at one position (residue 294) a conservative substitution (Lys→Gln) was present. Using a high-titer phage preparation from a clone binding 45B1, the phage particles competed for binding to native PyAMA1 in a concentration-dependent manner (data not shown). We then synthesized a cyclized synthetic peptide called PEP45B1 (Fig. 1A) based on the sequence in phage clones 1 and 2 flanked by cysteine residues, which also contained a GlyGlyGlySer tail at the carboxyl terminus to mimic the form of the peptide presented on the phage. In a competition ELISA, PEP45B1 competed for the binding of MAB 45B1 to native PyAMA1 in a concentra-
FIG. 1. Identification of mimotopes for MAb 45B1. (A) Alignment of the *P. yoelii* YM AMA1 protein sequence with the deduced 7-mer sequences from phage clones that bound to the MAb. The sequence from residues 290 to 296 in AMA1 is most closely related to the phage sequences. The cysteine residues at positions 282 and 354 form a disulfide bond. Significant identities are shaded and indicated (:). The Gln (Q) residue 294 in AMA1 is replaced by the conservative substitution of Lys (K), in all of the peptide sequences (:). Phage clones 1 and 2 contained identical nucleotide sequence (data not shown) and, together with clone 5, were the best match to the AMA1 sequence. PEP45B1 is the sequence of a cyclized synthetic peptide corresponding to the mimotope such that it adopts a conformation that mimics the epitope such that it adopts a conformation that mimics the 45B1 epitope on PyAMA1.

Ten phage clones selected from the constrained peptide library with MAb 25.77 were also sequenced, but no consensus sequence was identified (five clones had the sequence FYEMPTR, three had the sequence DHFGMHR, one had the sequence TPTFRAN, and one had the sequence TSLWALQ). When these sequences were compared to the known P235 protein sequences (15, 25, 31, 45) and *P. yoelii* genome sequence data [http://www.plasmodb.org], no region of similarity was identified.

The MAb 45B1 epitope is conserved and located within the AMA1 domain II loop. AMA1 domains I and II consist of a pair of PAN domains, structures found in proteins with diverse adhesion functions (54). Within the pair of PAN domains there are six loops that extend from the PAN scaffold and surround a hydrophobic pocket (Fig. 2) (2, 39). The largest of these loops extends from the domain II PAN domain, and the 45B1 epitope is part of this domain II loop. The domain II loop initially packs against domain I in an extended conformation. It then forms a helix and a β-hairpin before forming a further region of extended conformation that returns to domain II. The 45B1 mimotope is in the first part of this loop (Fig. 2).

In the *P. falciparum* AMA1 crystal structure, the mimotope sequence (SASDQPK) runs along the surface of AMA1, packing against domain I (2). The side chains of the first two residues, serine and alanine, are completely buried, pointing toward domain I. Similarly, the aspartate and proline side chains point toward domain I. The side chains of the second serine and the lysine at the last position are exposed, pointing toward solvent. The side chain of the glutamine residue is partially exposed. The terminal carbonyl oxygen is buried, whereas the terminal amino group is exposed.

If antibody 45B1 bound to the SASDQPK sequence in the conformation observed in the *P. falciparum* structure, it would be expected that the exposed amino acids are selected for in the phage clones that bind to 45B1. The exposed second serine and the exposed lysine are selected for in all phage clones (Fig. 1A). Also, the structure is consistent with the selection of a lysine in place of a glutamine because only the amino group of the glutamine is exposed. Conversely, it would be expected that the side chains that are buried in the AMA1 structure would not be selected for in the 45B1-binding clones. The buried alanine is not selected in any phage clones. Also, the buried aspartate and proline residues are selected in all clones (Fig. 1A).

Presumably, these residues are required for maintaining the structure of the mimotope such that it adopts a conformation that mimics the conformation of the peptide in intact AMA1. Another possibility is that the SASDQPK motif adopts an alternative conformation that is recognized by 45B1. In fact, in the *P. vivax* AMA1 structure (39) only the SASQ residues were ordered, and it is likely that the PT residues adopt alternate conformations (discussed below).

The 45B1 constrained epitope within AMA-I is conserved. The 45B1 mimotope sequence (SASDQPK) is conserved and...
identical in most *Plasmodium* AMA1 sequences, including *P. falciparum* (6) (Fig. 1C). It differs at the last residue in *P. vivax* and the related parasites, *P. cynomolgi* and *P. fragile* (SASDQPT) (Fig. 1C). In *P. knowlesi* there is a conservative Lys→Arg substitution at the last position (SASDQPR) (Fig. 1C).

To examine the extent of conservation of the epitope recognized by MAb 45B1 within different *Plasmodium* species, subspecies, and isolates, parasitized erythrocytes were screened by IFA with MAb 45B1 and with MAb 28G2, an antibody that recognizes the conserved C terminus of AMA-1 (33) (Table 1). The epitope recognized by MAb 45B1 was detected in all subspecies and isolates of *P. yoelii*. The epitope was present in the primate parasite *P. knowlesi*, indicating that the Lys→Arg substitution is tolerated by 45B1. However, the epitope was not detected in *P. chabaudi*, *P. berghei*, or *P. falciparum* strains FCB-1 and 3D7. This result was surprising because in these parasites the SASDQPK sequence is conserved (Fig. 1C). Presumably, there are residues in addition to the SASDQPK sequence that are required for 45B1-binding.

**Passive immunization with MAb 45B1 and 25.77 protects against heterologous challenge in vivo.** It has been shown

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**TABLE 1.** Presence of the AMA1-specific MAb 28G2 and MAb 45B1 epitopes in *P. yoelii* subspecies and other *Plasmodium* species detected by immunofluorescence

<table>
<thead>
<tr>
<th>Parasite species, subspecies, or isolate</th>
<th>Recognition of MAb 28G2</th>
<th>Recognition of MAb 45B1</th>
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<tbody>
<tr>
<td><em>P. y. yoelii</em> YM</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. y. yoelii</em> 17X</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. y. yoelii</em> 33X</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. y. yoelii</em> 2CL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. y. yoelii</em> 2BR</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. y. yoelii</em> 3AE</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. y. yoelii</em> 1AR</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. y. killicki</em> 194ZZ</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. y. killicki</em> 193L</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. y. nigeriensis</em> N67</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. chabaudi</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. berghei</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. knowlesi</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. falciparum</em> FCB-1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. falciparum</em> 3D7</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+, present; −, absent.

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**FIG. 2.** Representations of the structure of domains I and II of *P. yoelii* AMA1 based on the *P. falciparum* AMA1 crystal structure (2). (A) Ribbon diagram of AMA1 highlighting the putative MAb 45B1 epitope (shown in green). Domain II is colored blue, and this view shows the domain II loop packing against domain I. (B and C) Space-filling representation of AMA1 showing surface residues, colored purple, that are not identical in the protein from *P. yoelii* YM and 17X lines. As with *P. falciparum*, amino acid substitutions are located on the polymorphic face (B), with no differences on the nonpolymorphic face (C). The MAb 45B1 epitope (green, panel C) is located at the center of the nonpolymorphic surface. (D) Stereo view of the 45B1 mimotope sequence within the *P. falciparum* AMA1 structure. Oxygen atoms are colored red; nitrogen blue and carbon atoms are green. Panels A and B are related by a 180° turn about the vertical axis. Lines and regions colored red (A to C) show the approximate locations and sizes of unstructured loops in the *P. falciparum* AMA1 crystal structure.
Previously that MAb 45B1 and 25.77 passively immunize mice against infection with *P. yoelii* YM. Although these MAb were raised to the YM line, they reacted with other isolates and subspecies of *P. yoelii* (Table 1). Therefore, it was of interest to determine whether they would protect against a heterologous blood-stage challenge infection, particularly a parasite such as the 17X line that is restricted to reticulocytes. Groups of six mice were passively immunized with either MAb 45B1 and 25.77 or a control antibody and then challenged with the avirulent *P. yoelii* 17X-infected red blood cells. The resultant parasitemia within both of the experimental groups was clearly attenuated relative to the control group (Fig. 3), indicating that both antibodies are effective.

**Passive immunization with a combination of the two protective MAb produces additive effects in vivo.** Although it has been shown that both MAb 45B1 and 25.77 passively immunize mice against infection with *P. yoelii* YM and 17X (Fig. 3), we wanted to test whether protection could be provided by a combination of these two antibodies against different targets, at a concentration of each antibody below that required to control parasitemia. A passive immunization dose escalation study was first performed with MAb 45B1 and 25.77 to determine the concentration of each antibody required to delay but not clear infection. Based on the results of such a study (data not shown), the total dosages to be administered in combination were set at 0.125 mg of 45B1 IgG and 0.25 mg of 25.77 IgG for each immunization. These amounts were four- to sixfold lower than that required to protect against a challenge infection when administered alone. The total amount of control IgG used was 0.375 mg, which is equivalent to the sum of both 45B1 and 25.77. After immunization and challenge with the virulent *P. yoelii* YM parasite, the resultant parasitemia in individual mice was recorded on alternate days, and the results are shown in Fig. 4. Passive immunization with 0.25 mg of MAb 25.77 increased the survival of mice by 2 days compared to controls. Passive immunization of mice with 0.125 mg of MAb 45B1 increased survival by 6 to 8 days compared to controls. After the coadministration of MAb 25.77 and 45B1, five of six mice survived the challenge infection, and only one mouse had to be sacrificed on day 16, 10 days after the controls had succumbed. All of the mice immunized with the control antibody succumbed to the infection at the same time as naive nonimmunized mice. Statistical analysis of the average parasitemia in each group on day 5 demonstrated significant differences between the experimental and control groups. The parasite densities for each group ranked from lowest to highest on day 5 were 25.77 + 45B1 < 45B1 < 25.77 < 48F8 (*P* < 0.05 for each group). These results show that the administration of a combination of a nonprotective dose of each MAb resulted in an additive effect.

**DISCUSSION**

In natural infection, protection against malaria develops as the result of exposure to the parasite, with a large repertoire of parasite proteins or variants thereof recognized by the immune system. A number of proteins have been identified as candidates for the development of malaria vaccines, and combinations of antigens from different stages of the life cycle have been proposed (29). However, there is little experimental evidence to support the concept that the use of several antigens would be more effective than the use of a single antigen. The study described in here provides evidence that a combination of antibodies to two blood-stage proteins is more effective than antibodies to a single protein. These results lend support to the potential of passive immunization to control malaria infection and suggest that a vaccine based on two or more blood-stage protein components might be more effective than one based on a single component.

Both 45B1 and 25.77 recognize the avirulent *P. yoelii* 17X line and attenuate the parasite infection in vivo. This parasite differs from the YM line in that it is restricted to growth in reticulocytes; therefore, the results indicate that both PyAMA1 and PyP235 are also involved in invasion of this second host cell type. It has been reported that MAb 25.77 had no activity against *P. yoelii* 17X in similar studies (18), and the basis for this difference in results is unknown. However, in the previous study when serum rather than purified IgG was used for passive immunization it is possible that the level of specific antibody achieved in the plasma was considerably less than that achieved here with IgG. The specific function of these two molecules during invasion of erythrocytes is the subject of much debate (2, 12, 22, 26). Both PyAMA1 and PyP235 have been shown to bind erythrocytes with different techniques (12, 35). Phage selected on MAb 25.77 had no apparent clear consensus sequence, and none of the peptide sequences could be identified in the known PyP235 sequences and those in the genome sequence database (http://www.plasmodb.org).

We have defined the epitope on AMA1 for 45B1, an MAb that can protect against a blood-stage infection. The antibody binds to a cyclized peptide sequence that is almost identical to residues 290 to 296 located in domain II of the PyAMA1 ectodomain. As shown by IFA, the epitope for this MAb is also expressed by other isolates or subspecies of *P. yoelii*, as well as by the primate malaria parasite *P. knowlesi*. In *P. knowlesi* the
putative 45B1 epitope is identical except for the conservative substitution of the lysine with an arginine in the last position of the mimotope sequence (SASDQPR). Interestingly, the sequence SASDQPK is conserved in the AMA1 proteins of *P. falciparum*, *P. chabaudi*, and *P. berghei*, which do not bind the antibody. Presumably, there are other amino acid substitutions in the vicinity of this sequence that prevent 45B1 binding to the *P. falciparum* and *P. chabaudi* proteins, and these differences are not present in the *P. knowlesi* AMA1. This result is surprising because the *P. yoelii* and *P. chabaudi* AMA1 sequences are similar (75% identity), whereas *P. knowlesi* AMA1 and *P. falciparum* AMA1 are more divergent (55 and 50% identities with PyAMA1, respectively). Close examination of the residues surrounding the SASDQPK motif in the AMA1 structure did not reveal any obvious areas where *P. yoelii* and *P. knowlesi* are similar and *P. falciparum*, *P. chabaudi*, and *P. berghei* differ (data not shown).

A similar region within PfAMA1 domain II was reported to be recognized by a *P. falciparum* inhibitory antibody MAb 4G2 (39). Alanine scanning mutations within the domain II loop were found to disrupt 4G2 binding. For example, replacing the D or K in the mimotope sequence (SASDQPK) with alanine disrupted 4G2 binding. This finding, together with the results presented here, provides strong evidence for the importance of the domain II loop as a target for protective immunity and provides indirect evidence for this region being critical to AMA1 function.

There are several lines of evidence to suggest that the AMA1 domain II loop is highly mobile, adopting different conformations, and, intriguingly, this mobility may be required for AMA1 function. Brief treatment of AMA1 with protease resulted in the cleavage of the domain II loop (16). This would suggest that this region of AMA1 is mobile and solvent accessible. In addition, in the *P. vivax* crystal structures electron density for the domain II loop was not observed (56). Presumably, the domain II loop can adopt different conformations such that the different conformations could not be resolved in the electron density. In the *P. falciparum* structure the domain II loop was observed (2). It is most likely that crystal packing contacts locked the domain II loop into one of its preferred conformations. More precisely, within the SASDQPK 45B1 mimotope sequence, the SASDQ region has an identical con-

![Graph showing the course of P. yoelii YM infection in individual BALB/c mice within groups of six passively immunized with either MAb 48F8, MAb 25.77, MAb 45B1, or a mixture of 45B1 and 25.77. After challenge with parasites the parasitemia was monitored by microscopy on alternate days. An asterisk denotes the day that one mouse was sacrificed.](http://iai.asm.org/)
formation in the *P. vivax* and *P. falciparum* structures, but the PK motif (PT in *P. vivax*) was disordered in the *P. vivax* structure. The 45B1 epitope therefore spans a region that is conformationally ordered and a region that is conformationally mobile. However, 45B1 almost certainly only recognizes one conformation. Presumably, 45B1 is able to inhibit AMA1 function by fixing the domain II loop into this one conformation. This would imply that the mobility of the domain II loop and the ability of the loop to adopt different conformations are critical to AMA1 function.

It is currently not clear how the domain II loop contributes to AMA1 function. One possibility is that the domain II loop is required for completing the extended hydrophobic pocket between the AMA1 loops. Two of the hydrophobic residues that make up this pocket are part of the domain II loop, and it is speculated that this is the AMA1 receptor binding site (2). Another possibility is that the domain II loop binds directly to red blood cells. A synthetic peptide, 4337 (loop II residues 393 to 473), selectively bound to red blood cells (9). Intriguingly, in isolation this peptide adopted a helical conformation, whereas in the *P. falciparum* crystal structure this region adopts an extended conformation. These results are consistent with the hypothesis that different conformations of the domain II loop are required for AMA1 function.

Earlier studies demonstrated that natural human immune responses competed for binding AMA1 specific MAb 4G2 (D. L. Narum and A. W. Thomas, unpublished results). Most importantly, the consistency between the results reported by Pizarro et al. (39) and those reported here show that, despite the potential for significant polymorphisms, a conserved region of AMA1 is susceptible to inhibitory antibodies. With respect to PyP235, we previously reported that the frequency of antigenic variation of P235 in *P. yoelii* YM was low (<0.01%) based on screening with a PyP235 specific MAb panel (31). A conserved role of P235 in erythrocyte invasion by both PyYM and Py17X suggests the functional domain(s), yet unidentified, is conserved. This may be important for the vaccine development of proteins identified as part of the reticulocyte binding-like superfamily in *P. vivax* (14, 24) and in *P. falciparum* (41, 42, 49, 51, 55).

The observed additive effect of antibodies to the two different proteins on protection is significant in the context of the development of multicomponent vaccines against the erythrocytic stage of human malaria. A vaccine that targets multiple malaria proteins may induce immune responses that act additively or possibly synergistically, although this has been difficult to show in human populations in areas of endemicity (5, 19), as well as in a primate model (48). AMA1 is an important vaccine candidate for both *P. falciparum* and *P. vivax*, and the conserved AMA1 family has been shown to be protective when used to immunize against challenge infections with the primate malarial species (8, 11) and the rodent malarial species (1, 32). A recombinant PfAMA1 phase I human vaccine trial was recently completed (28), and another has been initiated (Malaria Vaccine Development Branch). The *P. yoelii* P235 rhoptry protein family is protective as a vaccine against the blood-stage infection (18) and is part of a superfamily in the *Plasmodium* genus. These and other vaccine candidates, including MSP1 (34, 47) and the erythrocyte binding antigen 175 (20), are potential components of such a multicomponent vaccine. Establishing the rationale for future combinations will be difficult, although it is clearly warranted.

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