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# Merozoite Surface Protein 4/5 Provides Protection against Lethal Challenge with a Heterologous Malaria Parasite Strain

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**Immunization with merozoite surface protein 4/5 (MSP4/5), the murine malaria homologue of *Plasmodium falciparum* MSP4 and MSP5, has been shown to protect mice against challenge by parasites expressing the homologous form of the protein. The gene encoding MSP4/5 was sequenced from a number of *Plasmodium yoelii* isolates in order to assess the level of polymorphism in the protein. The gene was found to be highly conserved among the 13 *P. yoelii* isolates sequenced, even though many of the same isolates showed pronounced variability in their $MSP1_{19}$ sequences. Nonsynonymous mutations were detected only for the isolates *Plasmodium yoelii nigeriensis* N67 and *Plasmodium yoelii killicki* 193L and 194ZZ. Immunization and challenge of BALB/c mice showed that the heterologous MSP4/5 proteins were able to confer a level of protection against lethal *Plasmodium yoelii yoelii* YM challenge infection similar to that induced by immunization with the homologous MSP4/5 protein. To explore the limits of heterologous protection, mice were immunized with recombinant MSP4/5 protein from *Plasmodium berghei* ANKA and *Plasmodium chabaudi adami* DS and challenged with *P. y. yoelii* YM. Interestingly, significant protection was afforded by *P. berghei* ANKA MSP4/5, which shows 81% sequence identity with *P. y. yoelii* YM MSP4/5, but it was abolished upon reduction and alkylation. Significant protection was not observed for mice immunized with recombinant *P. c. adami* DS MSP4/5, which shows 55.7% sequence identity with *P. y. yoelii* YM MSP4/5. This study demonstrates the robustness of MSP4/5 in conferring protection against variant forms of the protein in a murine challenge system, in contrast to the situation found for other asexual-stage proteins, such as $MSP1_{19}$ and AMA1.**

Malaria is the most important parasitic disease infecting humans, resulting in ~300 to 500 million clinical cases and 1 to 3 million deaths per year (30). *Plasmodium falciparum* is responsible for the most severe and fatal form of malaria. Due to the emergence of drug-resistant malaria parasites and insecticide-resistant mosquito vectors, there is an urgent need to develop a vaccine capable of reducing both morbidity and mortality from infection, as well as the transmission of drug-resistant malaria parasites.

Twenty years of antigen identification and characterization has yielded many potential vaccine candidates (26), but there is still no vaccine against human malaria. A recognized problem is the high level of polymorphism of antigens that are targets of an antimalarial immune response, especially those exposed on the surface of the merozoite and the infected red blood cell (3, 7, 13). Indeed, in rodent malaria challenge studies, two leading vaccine candidates, $MSP1_{19}$ (28, 29) and AMA1 (10), were found to protect from homologous but not heterologous challenge. The ability to confer protection against infection by parasites expressing variant forms of an antigen would be the hallmark of a robust vaccine that would have an extended life span.

Two recently characterized *P. falciparum* merozoite surface proteins, MSP4 (22) and MSP5 (35), are candidates for inclusion in a blood stage malaria vaccine. We have identified the rodent malaria homologue of these genes (MSP4/5) (4), and all the predicted proteins show structural similarities, including an N-terminal signal sequence, a C-terminal glycosylphosphatidylinositol (GPI) anchor, and a single epidermal growth factor (EGF)-like domain (4, 16). A membrane-bound, surface-exposed location on the merozoite has been demonstrated by Triton X-114 partitioning and immunofluorescence (4, 16).

In contrast to many blood stage antigen genes, *msp4* and *msp5* show limited sequence diversity. The mature protein of *msp5* has no reported diversity among the *P. falciparum* isolates examined (35). Only limited antigenic diversity for MSP4 has been detected, with nine residues exhibiting polymorphism (34). It is not known whether this conservation is due to functional constraints or a lack of immune (or other) selection pressure.

Recent studies utilizing recombinant MSP4/5 expressed in *Escherichia coli* have demonstrated its efficacy in protecting BALB/c mice against a lethal *Plasmodium yoelii yoelii* YM blood stage challenge (17, 19). The present study aimed to extend these findings by determining the ability of MSP4/5 to confer protection against heterologous challenge. We first determined the level of polymorphism present in the sequence of MSP4/5 from various rodent malaria isolates and expressed variant sequences as recombinant proteins in *E. coli*. Mice were immunized, and antibodies were used to determine cross-reactivity by Western blotting and enzyme-linked immunosorbent assay (ELISA) against the heterologous proteins. The protective efficacies of the variant proteins compared to that of *P. y. yoelii* YM MSP4/5 were determined by challenging immunized mice with the lethal parasite line *P. y. yoelii* YM. To

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TABLE 1. Primers used in this study for PCR amplification of *msp4/5* from various *P. yoelii* isolates

| Primer | Sequence[a] |
|---|---|
| p623 | 5′ GTTAATATATCATGGAAACTAAAA 3′ |
| p624 | 5′ AATCGCCATTTGTATTACCTGGTTGTACTGAAGGTT 3′ |
| p625 | 5′ AACCTTCAGTACAACCAGGTAATACAAATGGCGATT 3′ |
| p626 | 5′ GGAAGATCTTTAATGATGATGATGATGATGTGAATGCGCACTGAGTAATTCG 3′ |
| p648 | 5′ AAAAAAATGAAAATCACAAATTAC 3′ |
| p1106 | 5′ GGCATGCCATGGTTCATGAAACTTCTTTAA 3′ |

[a] Restriction endonuclease recognition sites are underlined.

explore the limits of heterologous protection, mice were immunized with MSP4/5 from the rodent malaria parasite species *Plasmodium berghei* ANKA and *Plasmodium chabaudi adami* DS, which show 81 and 55.7% sequence identity with MSP4/5 of the *P. y. yoelii* YM challenge strain, respectively.

## MATERIALS AND METHODS

**Experimental animals, parasites,and genomic DNA.** Female BALB/c mice aged 6 to 8 weeks were purchased from Central Animal Services (Monash University, Victoria, Australia). Michael Good (Queensland Institute of Medical Research, Brisbane, Australia) kindly provided the *P. y. yoelii* YM and 17XNL parasites. *P. y. yoelii* 1AR, *P. y. yoelii* 2BG, and *Plasmodium yoelii killicki* 193L parasite stabilates were kindly provided by David Walliker (University of Edinburgh, Edinburgh, Scotland). *P. y. yoelii* 265BY genomic DNA was kindly provided by Laurent Renia (INSERM, CHU Pitie-Salpetriere, Paris, France). Genomic DNAs from the *P. yoelii* isolates 193L, 194ZZ, N67, 1AR, 2BR, 2CL, 33X, 3AE, 17XB, and 17XS were prepared as described previously (2). The primary isolates were obtained from thicket rats (*Thamnomys rutilans*) at different locations and times (2), with the exception of *P. y. yoelii* 17XB and 17XS, which are avirulent lines derived from cloning of 17XL.

**Sequencing of MSP4/5 from various *P. yoelii* isolates and cloning into an *E. coli* expression vector.** Sequencing was performed using the PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. Sequencing reaction products were separated on a model 373 automated DNA sequencer (Applied Biosystems, Branchburg, N.J.). PCRs were performed using Ampli-Taq Gold (Applied Biosystems, Branchburg, N.J.), and the PCR products were directly sequenced on both strands. When polymorphisms were identified, direct sequencing of PCR products was repeated to confirm the sequence. The sequences were analyzed by Sequencher software version 3.1.1 (Gene Codes Corp., Ann Arbor, Mich.), and alignments were compiled using Eclustalw (WebANGIS; The Australian Genomic Information Centre, The University of Sydney, Sydney, New South Wales, Australia).

For recombinant-protein expression, all genes lacking the predicted signal and GPI anchor sequence were cloned into the pTrcHisA vector (Invitrogen Corp., Carlsbad, Calif.) as previously described (16). Primers p648 and p623 (Table 1) were used to amplify *msp4/5* from genomic DNAs of the various *P. yoelii* isolates. These primers were designed from the sequence encoding the N-terminal signal and C-terminal GPI anchor sequences of *P. y. yoelii* YM MSP4/5. PCRs were performed under the following conditions: one cycle of 94°C for 3 min; 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and one cycle of 72°C for 5 min. Splice overlap extension PCR (12) was performed on *P. y. killicki* 193L and *Plasmodium yoelii nigeriensis* N67 with primers p623, p624, p625, and p648 (Table 1) to remove the intron. PCR with primers p626 and p1106 (Table 1) was then used to remove the predicted signal and anchor sequences from the original PCR product before cloning it into pUC18 and sequencing it. A clone with the correct sequence was selected, digested with NcoI/BglII, and cloned into NcoI/BglII-digested pTrcHisA. Error-free recombinant plasmids were then transformed into *E. coli* BL21(DE3) (Novagen, Milwaukee, Wis.) for protein expression.

**Recombinant six-His-tagged fusion protein expression and purification.** Purification of six-His-tagged proteins was conducted using TALON Metal Affinity Resin (Clontech, Palo Alto, Calif.) as described previously (16). Protein purity and integrity were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gels were stained with Coomassie brilliant blue. The protein concentration was determined using the Bio-Rad (Hercules, Calif.) protein assay following the manufacturer's instructions. When required, proteins were concentrated to the required volume using an Ultrafree-15 centrifugal filter device (Millipore Corp., Bedford, Mass.) with a 10,000-Da molecular mass cutoff and stored at −80°C.

**Vaccination and challenge infection.** Groups of female BALB/c mice were immunized with 25 μg of recombinant MSP4/5 protein emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) administered intraperitoneally (i.p.). Two subsequent boosters of antigen emulsified in incomplete Freund's adjuvant (Difco Laboratories) were delivered i.p. at monthly intervals. Control mice were injected with phosphate-buffered saline (PBS) emulsified in the appropriate Freund's adjuvant. Sera were collected before the initial injection (preimmunization sera) and 2 days before challenge (prechallenge sera). Twelve to 14 days after the final injection, the mice were challenged i.p. with $10^5$ *P. y. yoelii* YM-parasitized red blood cells. Parasitemia was monitored microscopically by Giemsa-stained thin blood smears, and the percent parasitemia was determined by counting 500 cells per slide.

**Immunochemical assays.** Antibody responses were determined by ELISA as previously described (33) using *E. coli*-derived *P. y. yoelii* YM MSP4/5 as the coating antigen. Western blotting was performed as previously described (16) using the various *E. coli*-derived MSP4/5 proteins.

**Statistics.** All statistical analyses were carried out using GraphPad (San Diego, Calif.) Prism software. To determine significant differences in survivor outcome between groups, Fisher's exact test was performed. For determining significant differences in peak parasitemia or prechallenge antibody levels, the Mann-Whitney U test was performed. The Spearman rank correlation test was used to check the association between optical density values of prechallenge sera and peak parasitemia and between percent amino acid identity and peak parasitemia, as well as between percent amino acid identity and percent survival.

**Nucleotide sequence accession numbers.** The nucleotide sequence data reported in this paper are available in the GenBank database under the accession numbers AY563008 to AY563020.

## RESULTS

**Sequence analysis of *msp4/5* proteins from various *P. yoelii* isolates.** The gene encoding MSP4/5 was sequenced in a number of *P. yoelii* isolates in order to assess the level of polymorphism in the gene. Deduced amino acid sequences (lacking the N-terminal signal and C-terminal GPI anchor sequences) were compared by an Eclustalw alignment (Fig. 1). Nonsynonymous mutations relative to *P. y. yoelii* YM were observed only for the isolates *P. y. nigeriensis* N67, *P. y. killicki* 193L, and *P. y. killicki* 194ZZ. *P. y. killicki* 193L and 194ZZ have the same $MSP1_{19}$ sequence, and the MSP4/5 sequence was found to be identical. The most variant forms were *P. y. killicki* 193L and 194ZZ, with eight nonsynonymous mutations and two synonymous mutations, and *P. y. nigeriensis* N67, with nine nonsynonymous mutations and four synonymous mutations. There was a single synonymous point mutation detected in the EGF-like domains of *P. y. yoelii* 17XB, 3AE, and 2BR.

All amino acid sequence variations observed in MSP4/5 were due to point mutations, except for one case in which an aspartic acid residue was inserted into the aspartic acid-glutamic acid repeat region of *P. y. nigeriensis* N67. Amino acid substitutions were clustered in the N terminus of MSP4/5, whereas the EGF-like domain was in general conserved, except

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YM      23 VHEISLINTKKYQRNIFCAKRILGDPPSANPSTSLNQNEDNHNSNPDANKGNTNKSDKLNGDNIPNSQSKSENTNLSESPKTGTASNPPQPEPSVQ 118
193L    23 ...T.....................T...S.....................................G...........S..... 118
194ZZ   23 ...T.....................T...S.....................................G...........S..... 118
N67     23 ...T.....................T...S.........S.......K...................S..... 118
1AR     23 ..... 118
2BG     23 ..... 118
265BY   23 ..... 118
2BR     23 ..... 118
2CL     23 ..... 118
33X     23 ..... 118
3AE     23 ..... 118
17XB    23 ..... 118
17XNL   23 ..... 118
17XS    23 ..... 118

YM      119 PGNTNGDSQNSIHSDDEEEDDEDEDDDE-ED**CSVNNGGCGENLLCEKMESGIIKCSCPSGYKLNGTSC**IELLSAHS 193
193L    119 .....P.H.....E..-..... 193
194ZZ   119 .....P.H.....E..-..... 193
N67     119 .....DD.....N..... 194
1AR     119 .....~..... 193
2BG     119 .....-..... 193
265BY   119 .....-..... 193
2BR     119 .....-..... 193
2CL     119 .....-..... 193
33X     119 .....-..... 193
3AE     119 .....-..... 193
17XB    119 .....~..... 193
17XNL   119 .....-..... 193
17XS    119 .....-..... 193
```

FIG. 1. Alignment of predicted MSP4/5 protein sequences from various *P. yoelii* isolates and clones. The full sequence comprising the extracellular domain from residues 23 to 193 is shown for *P. y. yoelii* YM, the parasite line that was used for all challenge experiments. The EGF-like domain is indicated in boldface. For the other parasite proteins, identical residues are indicated by dots and amino acids differing from those of *P. y. yoelii* YM are indicated.

for *P. y. nigeriensis* N67, in which there is a residue substituted in the EGF-like domain. In all cases, the six cysteine residues in the EGF-like domain were completely conserved.

Sequence variation was also observed in the intron with five single-point mutations, a 1-bp deletion, and a 16-bp deletion observed in *P. y. killicki* 193L and 194ZZ. There were seven point mutations and a 1-bp deletion detected for *P. y. nigeriensis* N67. *P. y. yoelii* 1AR and 2BG have identical 29-bp insertions in the intron but no other sequence variation. The introns of the remainder of the strains are completely conserved.

**Analysis of recombinant MSP4/5 proteins from various rodent malaria isolates expressed in *E. coli*.** The MSP4/5 proteins from *P. y. yoelii* YM, *P. y. killicki* 193L, *P. y. nigeriensis* N67, *P. berghei* ANKA, and *P. c. adami* DS were expressed as recombinant His-tagged proteins in *E. coli*. The purified proteins were analyzed by Western blotting with an anti-His antibody and sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing and nonreducing conditions, and each generally had a unique pattern of bands (data not shown). *P. y. yoelii* YM and *P. y. killicki* 193L MSP4/5 were exceptions, as they had identical banding patterns. Under reducing conditions, *P. y. yoelii* YM and *P. y. killicki* 193L MSP4/5 both migrate at ~36 kDa. *P. y. nigeriensis* N67 and *P. berghei* ANKA MSP4/5 migrate at just below 36 kDa. *P. c. adami* DS MSP4/5 migrates at ~40 kDa.

In order to ascertain the comparative immunoreactivities of the variant MSP4/5 proteins, immunoblotting was performed using polyclonal sera raised to the recombinant proteins on reducing (Fig. 2) and nonreducing (data not shown) gels. Antibodies raised to recombinant *P. y. yoelii* YM, *P. y. killicki* 193L, and *P. y. nigeriensis* N67 MSP4/5 cross-reacted strongly with each other. The reactivity of each of the sera with recombinant *P. berghei* ANKA MSP4/5 was reduced, while reactivity was absent for recombinant *P. c. adami* DS MSP4/5, except for the *P. y. yoelii* YM antisera, which showed low levels of reactivity. Antibodies raised to recombinant *P. berghei* ANKA MSP4/5 weakly recognized *P. y. yoelii* YM and *P. y. nigeriensis* N67 MSP4/5, but not *P. y. killicki* 193L or *P. c. adami* DS MSP4/5. Antibodies raised to recombinant *P. c. adami* DS MSP4/5 faintly recognized *P. berghei* ANKA, *P. y. nigeriensis* N67, and *P. y. killicki* 193L MSP4/5, but not *P. y. yoelii* YM MSP4/5. The band migrating at 30 kDa has been determined to be a contaminating *E. coli* protein present in all purifications (16).

Under nonreducing conditions, broad cross-reactivity was observed among all recombinant proteins (data not shown). This broad cross-reactivity was also observed by ELISA when prechallenge sera were tested against yeast-derived *P. y. yoelii* YM MSP4/5 (19) and when serum raised against *P. y. yoelii* YM parasites was reacted with the various recombinant MSP4/5 proteins (data not shown).

**Immunization of mice with variant MSP4/5 proteins and challenge with *P. y. yoelii* YM.** Groups of BALB/c mice were immunized with three doses of PBS (control) or 25 μg of recombinant MSP4/5-His protein emulsified in adjuvant at monthly intervals. Two weeks after the final immunization, the

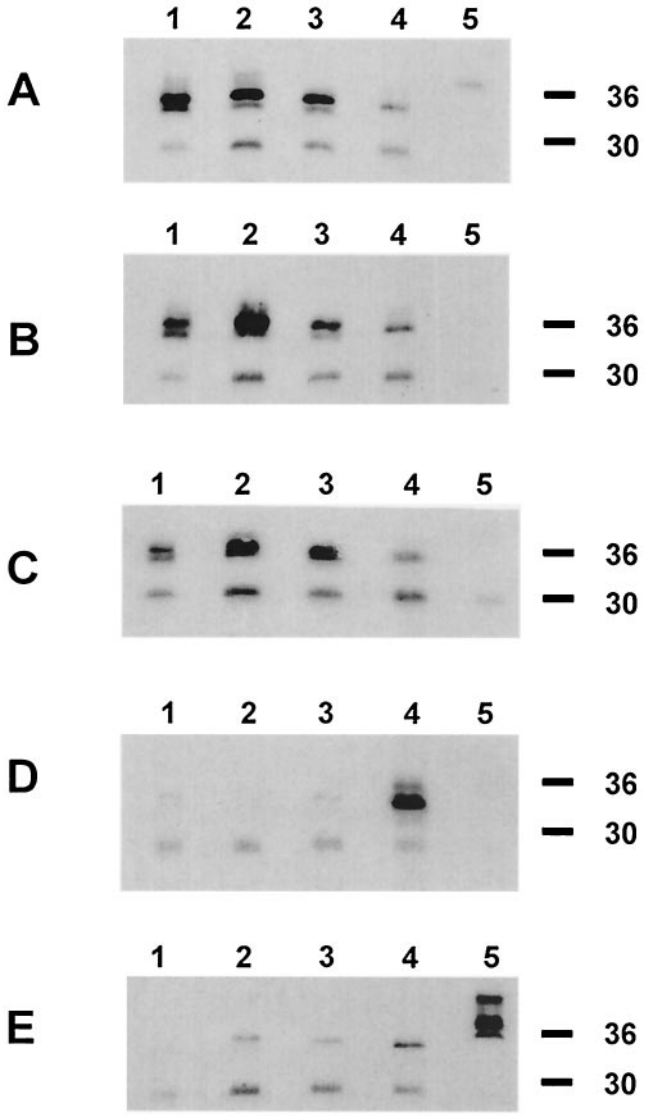


FIG. 2. Immunoblot analysis of purified *E. coli*-derived MSP4/5-His proteins from *P. y. yoelii* YM (lane 1), *P. y. killicki* 193L (lane 2), *P. y. nigeriensis* N67 (lane 3), *P. berghei* ANKA (lane 4) and *P. c. adami* DS (lane 5). The proteins were reduced prior to being loaded. The membrane was probed with mouse antisera diluted 1:2,500 raised against *P. y. yoelii* YM (A), *P. y. killicki* 193L (B), *P. y. nigeriensis* N67 (C), *P. berghei* ANKA (D), and *P. c. adami* DS (E) MSP4/5. Molecular mass standards (in kilodaltons) are on the right.

mice were challenged with $10^5$ *P. y. yoelii* YM-parasitized red blood cells, and the percent parasitemia was determined daily starting at day 3. Six separate vaccination trials were performed (Fig. 3), and they are summarized in Tables 2 and 3. Statistical comparisons of survival outcome using Fisher's exact test and peak parasitemias using the Mann-Whitney test are summarized in Table 4.

There was a statistically significant inverse correlation between percent amino acid identity and peak parasitemia (Spearman rank correlation coefficient; $r = -0.9747$; $P = 0.0167$). When survival for each challenge was considered, there was a statistically significant correlation between percent amino acid identity and percent survival (Spearman rank correlation test; $r = 0.8053$; $P < 0.0001$).

**Analysis of antibody responses of immunized mice.** The prechallenge antibody responses of the control and recombinant-protein-immunized mice were determined by ELISA using recombinant *P. y. yoelii* YM MSP4/5 protein as the target (Fig. 4). The Mann-Whitney test was used to determine statistical significance between antibody responses. There was a statistically significant difference in antibody response between the control mice and the *P. y. yoelii* YM, *P. y. killicki* 193L, *P. y. nigeriensis* N67, *P. berghei* ANKA, and *P. c. adami* DS MSP4/5-immunized mice. For the first three trials (analyzed separately), there was no statistically significant difference among antibody responses for the *P. y. yoelii* YM, *P. y. killicki* 193L, and *P. berghei* ANKA MSP4/5-immunized mice. Statistically significant differences were evident for trial 4. *P. y. yoelii* YM MSP4/5-immunized mice had a statistically significantly higher antibody response than mice immunized with *P. y. nigeriensis* N67, *P. berghei* ANKA, and *P. c. adami* DS MSP4/5. For trial 5, there were no statistically significant differences in antibody levels. For trial 6, the antibody levels of mice immunized with *P. berghei* ANKA, reduced and alkylated *P. berghei* ANKA, and *P. c. adami* MSP4/5 were significantly lower than those of the *P. y. yoelii* YM MSP4/5-immunized mice.

There was a statistically significantly higher prechallenge antibody response in the surviving mice immunized with *P. berghei* ANKA ($P = 0.0422$) and *P. c. adami* ($P = 0.0203$) MSP4/5.

There was a statistically significant inverse correlation between the antibody level and peak parasitemia for *P. y. yoelii* YM ($r = -0.3442$; $P = 0.0429$), *P. berghei* ANKA ($r = -0.5558$; $P = 0.0048$), and *P. c. adami* DS ($r = -0.6670$; $P = 0.0025$) MSP4/5-immunized mice, but not for *P. y. killicki* 193L, or *P. y. nigeriensis* N67 MSP4/5-immunized mice.

## DISCUSSION

Limited sequence diversity was identified in the *msp4/5* genes from 13 *P. yoelii* isolates, even though many of the same isolates differ markedly in their $MSP1_{19}$ sequences (ranging from 2 to 22% in a 98-amino-acid stretch) (2). Nonsynonymous mutations were detected only for the isolates *P. y. killicki* 193L and 194ZZ and *P. y. nigeriensis* N67. Compared to *P. y. yoelii* YM MSP4/5, the greatest variations in sequence were detected for *P. y. killicki* 193L and 194ZZ and *P. y. nigeriensis* N67. *P. y. killicki* 193L and 194ZZ MSP4/5 differs from that of *P. y. yoelii* YM at 8 amino acids out of 171 (4.7%), whereas *P. y. nigeriensis* N67 MSP4/5 differs from that of the challenge strain at 9 amino acid positions out of 171 (5.3%). The $MSP1_{19}$ sequences of these strains differ from that of the challenge strain by 14 out of 98 (14.3%) and 22 out of 98 (22.4%) amino acids, respectively. As determined for several other antigens of *Plasmodium* spp., there were more nonsynonymous than synonymous mutations observed (3, 13).

While the mature MSP5 protein was found to be completely conserved across a number of isolates (35), the sequence variations identified in MSP4/5 and MSP4 were found to be similar. In both cases, they is located in the N-terminal region of the proteins, are due to point mutations, and occur at similar rates (overall, 3.3% for MSP4) (34). Only one amino acid

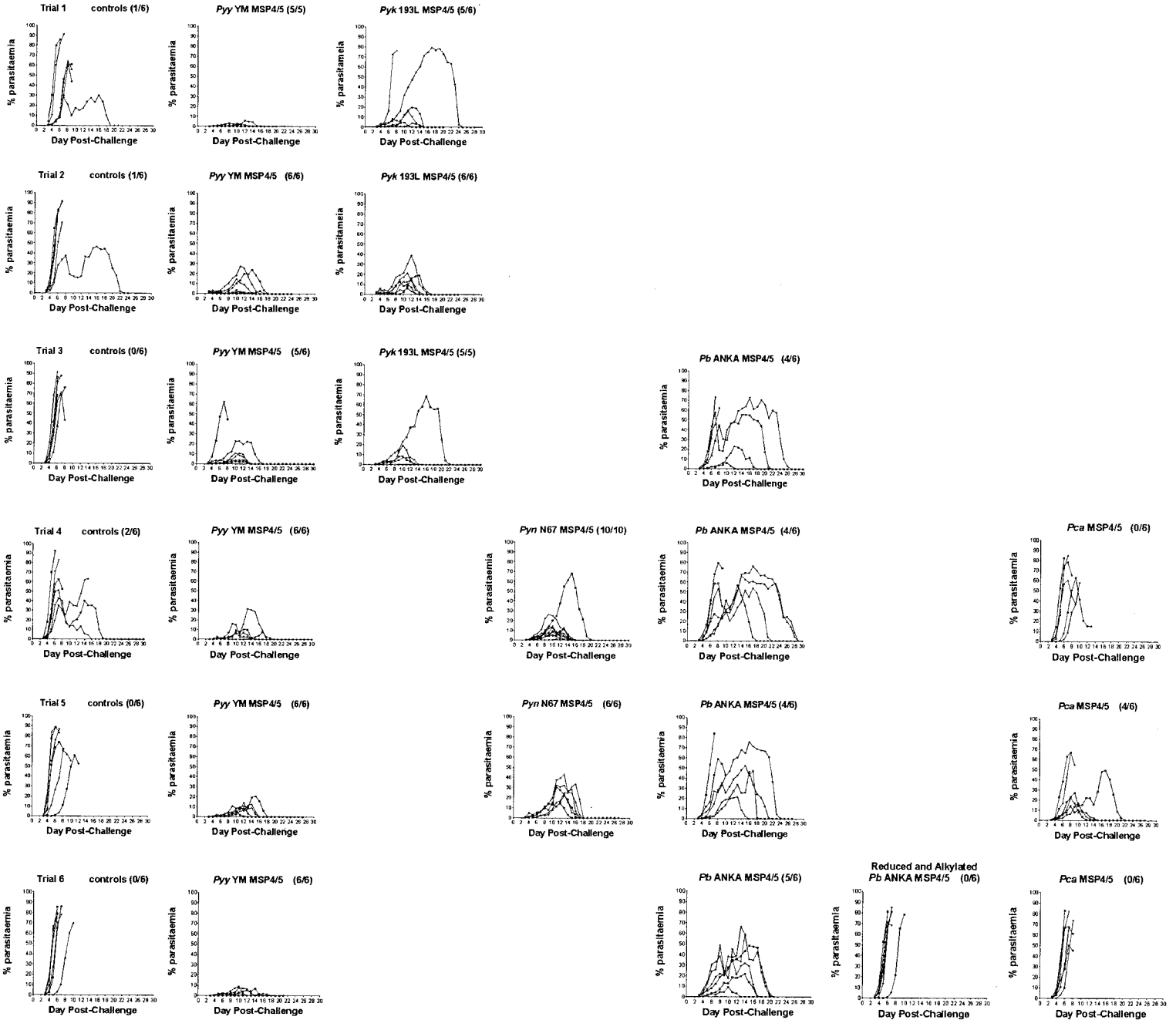
Trial 1 controls (1/6)
Pyy YM MSP4/5 (5/5)
Pyk 193L MSP4/5 (5/6)
Trial 2 controls (1/6)
Pyy YM MSP4/5 (6/6)
Pyk 193L MSP4/5 (6/6)
Trial 3 controls (0/6)
Pyy YM MSP4/5 (5/6)
Pyk 193L MSP4/5 (5/5)
Pb ANKA MSP4/5 (4/6)
Trial 4 controls (2/6)
Pyy YM MSP4/5 (6/6)
Pyn N67 MSP4/5 (10/10)
Pb ANKA MSP4/5 (4/6)
Pca MSP4/5 (0/6)
Trial 5 controls (0/6)
Pyy YM MSP4/5 (6/6)
Pyn N67 MSP4/5 (6/6)
Pb ANKA MSP4/5 (4/6)
Pca MSP4/5 (4/6)
Trial 6 controls (0/6)
Pyy YM MSP4/5 (6/6)
Pb ANKA MSP4/5 (5/6)
Reduced and Alkylated
Pb ANKA MSP4/5 (0/6)
Pca MSP4/5 (0/6)
% parasitaemia
Day Post-Challenge

FIG. 3. Blood-stage parasitemia in groups of mice in trials 1 to 6. Each trial is displayed horizontally, and groups immunized with identical preparations are aligned vertically. The number of survivors out of the total number of mice is shown in brackets next to the title of each graph. *Pyy*, *P. y. yoelii*; *Pyk*, *P. y. killicki*; *Pyn*, *P. y. nigeriensis*; *Pb*, *P. berghei* ANKA; *Pca*, *P. c. adami*.

change was identified in the EGF-like domain of MSP4/5 in a single *P. yoelii* isolate (*P. y. nigeriensis* N67). Similarly, only one amino acid change was identified in the EGF-like domain of MSP4 of *P. falciparum* (34).

Cross-reactivity in the antibody response to the recombinant proteins was explored by immunoblotting and ELISA. Under reducing conditions, immunoblots probed with polyclonal antisera raised to the recombinant MSP4/5 proteins showed strong cross-reactivity between *P. y. yoelii* YM, *P. y. killicki* 193L, and *P. y. nigeriensis* N67 MSP4/5 and marked differences in cross-reactivity for recombinant *P. berghei* ANKA and *P. c. adami* DS MSP4/5, which show 81 and 55.7% identity with that of the *P. y. yoelii* YM challenge strain, respectively (Fig. 2). The cross-reactivity observed among *P. y. yoelii* YM, *P. berghei* ANKA, and *P. c. adami* DS MSP4/5 is similar to that observed previously for parasite lysates (16) and appears to be related to the percent amino acid identity.

In contrast, broad cross-reactivity was observed by immunoblotting of the nonreduced proteins (data not shown). Similarly, broad cross-reactivity was apparent by ELISA, where prechallenge sera were tested against recombinant *P. y. yoelii* YM MSP4/5 protein (Fig. 4) or titrated on yeast-derived *P. y. yoelii* YM MSP4/5 (data not shown) and where convalescent phase sera raised in mice to a Triton X-114 detergent phase extract of *P. y. yoelii* YM parasites was titrated out on the recombinant MSP4/5 proteins (data not shown). Overall, the clear hierarchy of reactivity related to the primary amino acid sequence was not evident when nonreduced recombinant proteins were analyzed. The levels of cross-reactivity were very similar and did not appear to be consistently related to percent amino acid identity.

There was a statistically significant difference between survival outcomes in the control mice and mice immunized with the recombinant MSP4/5 proteins of *P. y. yoelii* YM, *P. y. killicki* 193L, *P. y. nigeriensis* N67, and *P. berghei* ANKA. This implies that immunization with any of the variant recombinant proteins, except that of *P. c. adami* DS, provides greater protection in terms of survival outcome than adjuvant alone. There was no statistically significant difference between survival outcomes in the *P. y. yoelii* YM, *P. y. killicki* 193L,and *P. y. nigeriensis* N67 MSP4/5-immunized mice, but survival was significantly reduced for *P. berghei* ANKA MSP4/5-immunized mice.

The effects of antigenic differences on protective efficacy were most pronounced with respect to peak parasitemia. *P. y. yoelii* YM MSP4/5-immunized mice had a peak parasite burden significantly lower than those of the *P. y. killicki* 193L and *P. berghei* ANKA MSP4/5-immunized mice, while there was no significant difference from that of *P. y. nigeriensis* N67 MSP4/5-immunized mice. *P. berghei* ANKA MSP4/5-immunized mice had a statistically significant higher peak parasite burden compared to *P. y. killicki* 193L and *P. y. nigeriensis* N67 MSP4/5-immunized mice, but perhaps surprisingly, it was not statistically significantly different from that of *P. c. adami* DS MSP4/5-immunized mice. The peak parasite burdens of *P. y. killicki* 193L and *P. y. nigeriensis* N67 MSP4/5-immunized mice were not significantly different.

Overall, there was a statistically significant inverse correlation between the percent amino acid identity and the peak parasitemia (Spearman rank correlation coefficient; $r$ =

TABLE 2. Summary of vaccination trials

| Trial no. | Immunizing antigen[a] | Peak parasitemia[b] | | | Survival (no. of survivors/ total no.) |
|---|---|---|---|---|---|
| | | Range (%) | Mean (%) | Days | |
| 1 | Controls | 30–91 | 66 | 6–16 | 1/6 |
| | *P. y. yoelii* YM MSP4/5 | 0–6 | 2 | 8–12 | 5/5 |
| | *P. y. killicki* 193L MSP4/5 | 4–79 | 33 | 8–19 | 5/6 |
| 2 | Controls | 47–92 | 78 | 6–16 | 1/6 |
| | *P. y. yoelii* YM MSP4/5 | 2–27 | 12 | 10–16 | 6/6 |
| | *P. y. killicki* 193L MSP4/5 | 6–39 | 19 | 9–14 | 6/6 |
| 3 | Controls | 68–92 | 80 | 6–8 | 0/6 |
| | *P. y. yoelii* YM MSP4/5 | 2–23 | 19 | 7–12 | 5/6 |
| | *P. y. killicki* 193L MSP4/5 | 0.2–69 | 21 | 10–16 | 5/5 |
| | *P. berghei* ANKA MSP4/5 | 6–73 | 49 | 7–16 | 4/6 |
| 4 | Controls | 42–92 | 66 | 6–15 | 2/6 |
| | *P. y. yoelii* YM MSP4/5 | 0.6–32 | 12 | 9–17 | 6/6 |
| | *P. y. nigeriensis* N67 MSP4/5 | 1–68 | 17 | 9–15 | 10/10 |
| | *P. berghei* ANKA MSP4/5 | 54–80 | 65 | 8–17 | 4/6 |
| | *P. c. adami* DS MSP4/5 | 58–85 | 71 | 6–10 | 0/6 |
| 5 | Controls | 61–89 | 78 | 6–11 | 0/6 |
| | *P. y. yoelii* YM MSP4/5 | 6–20 | 12 | 10–15 | 6/6 |
| | *P. y. nigeriensis* N67 MSP4/5 | 14–43 | 29 | 13–18 | 6/6 |
| | *P. berghei* ANKA MSP4/5 | 22–84 | 56 | 8–16 | 4/6 |
| | *P. c. adami* DS MSP4/5 | 14–67 | 39 | 8–18 | 4/6 |
| 6 | Controls | 70–86 | 80 | 6–10 | 0/6 |
| | *P. y. yoelii* YM MSP4/5 | 0–9 | 5 | 10–16 | 6/6 |
| | *P. berghei* ANKA MSP4/5 | 9–67 | 40 | 10–15 | 5/6 |
| | RA *P. berghei* MSP4/5 | 71–85 | 80 | 6–10 | 0/6 |
| | *P. c. adami* DS MSP4/5 | 50–83 | 71 | 6–8 | 0/6 |

[a] The amount of immunizing antigen was 25 μg. RA, reduced and alkylated.
[b] Number of parasites per 500 red blood cells counted; all mice were challenged with *P. y. yoelii* YM.

$-0.9747$; $P = 0.0167$). This suggests that with respect to the peak parasite burden, the heterologous recombinant MSP4/5 proteins are not as efficacious in controlling peak parasitemia as the homologous protein. There was also a statistically significant correlation between percent amino acid identity and percent survival (Spearman rank correlation coefficient; $r = 0.8053$; $P < 0.0001$). This suggests that with respect to survival outcome, the heterologous recombinant MSP4/5 proteins are not as efficacious in conferring protection as the homologous protein. Furthermore, as the recombinant proteins become more divergent in their primary amino acid sequences, the protective efficacy they afford decreases accordingly. Interestingly, there was a statistically significant inverse correlation between peak parasitemia and antibody level for *P. y. yoelii* YM, *P. berghei* ANKA, and *P. c. adami* DS MSP4/5-immunized mice but not for *P. y. killicki* 193L or *P. y. nigeriensis* N67 MSP4/5-immunized mice. There were also statistically significantly higher prechallenge antibody responses in the surviving mice immunized with *P. berghei* ANKA and *P. c. adami* MSP4/5, suggestive of antibody-mediated protective efficacy.

The near-complete conservation of the EGF-like domain of MSP4/5, MSP4, and MSP5 contrasts with findings reported for $MSP1_{19}$ and AMA1, where polymorphism exists in disulfide-bonded regions (11, 15, 23, 25, 31). This may be related to these conformational structures being critical for protection, as demonstrated by reduction and alkylation experiments (1, 20,

TABLE 3. Summary of relationships among percent amino acid identity, survival, and average peak parasitemia for mice immunized with various recombinant MSP4/5 proteins and challenged with *P. y. yoelii* YM

| Parameter | Value[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control mice | *P. y. yoelii* YM MSP4/5 immunized | *P. y. killicki* 193L MSP4/5 immunized | *P. y. nigeriensis* N67 MSP4/5 immunized | *P. berghei* ANKA MSP4/5 immunized | Reduced and alkylated *P. berghei* ANKA MSP4/5 immunized | *P. c. adami* MSP4/5 immunized |
| Amino acid identity with *P. y. yoelii* YM MSP4/5 (%) | N/A | 100 | 95.3 | 94.7 | 81 | 81 | 55.7 |
| No. survived/total | 4/36 | 34/35 | 16/17 | 16/16 | 17/24 | 0/6 | 4/18 |
| % Survival | 11 | 97 | 94 | 100 | 71 | 0 | 22 |
| Avg % peak parasitemia ± SEM | 74 ± 2.6 | 16 ± 2.9 | 24 ± 6.2 | 22 ± 4.3 | 53 ± 4.6 | 80 ± 2.0 | 60 ± 5.3 |

[a] N/A, not applicable.

TABLE 4. Statistical significance of differences in peak parasitemia and survival outcome of mice immunized with various recombinant MSP4/5 proteins and challenged with *P. y. yoelii* YM

| Immunizing MSP4/5 antigen | Parameter | Significance[c] | | | | |
|---|---|---|---|---|---|---|
| | | Control mice | *P. y. yoelii* YM MSP4/5 | *P. y. killicki* 193L MSP4/5 | *P. y. nigeriensis* N67 MSP4/5 | *P. berghei* ANKA MSP4/5 |
| *P. y. yoelii* YM | Peak parasitemia[a] | $P < 0.0001$ (SD) | | | | |
| | Survival[b] | $P < 0.0001$ (SD) | | | | |
| *P. y. killicki* 193L | Peak parasitemia | $P < 0.0001$ (SD) | $P = 0.0328$ (SD) | | | |
| | Survival | $P < 0.0001$ (SD) | $P = 1.5152$ (NSD) | | | |
| *P. y. nigeriensis* N67 | Peak parasitemia | $P < 0.0001$ (SD) | $P = 0.1093$ (NSD) | $P = 0.8854$ (NSD) | | |
| | Survival | $P < 0.0001$ (SD) | (NSD) | | | |
| *P. berghei* ANKA | Peak parasitemia | $P = 0.0001$ (SD) | $P < 0.0001$ (SD) | $P = 0.0013$ (SD) | $P = 0.0004$ (SD) | |
| | Survival | $P < 0.0001$ (SD) | $P < 0.0479$ (SD) | | | |
| Reduced and alkylated *P. berghei* ANKA | Peak parasitemia | $P = 0.9372$ (NSD) | $P = 0.0022$ (SD) | | | |
| | Survival | (NSD) | $P = 0.0022$ (SD) | | | |
| *P. c. adami* DS | Peak parasitemia | $P = 0.4522$ (NSD) | $P = 0.0003$ (SD) | $P = 0.0046$ (SD) | $P = 0.0017$ (SD) | $P = 0.1547$ (NSD) |
| | Survival | $P = 0.6581$ (NSD) | $P < 0.0001$ (SD) | | | $P = 0.0044$ (SD) |

[a] Statistical analysis of peak parasitemias was performed with the Mann-Whitney U test.
[b] Statistical analysis of survival outcome was performed with Fisher's exact test.
[c] NSD, no significant difference; SD, significant difference ($P < 0.05$).

21). In contrast, in homologous challenge studies, reduced and alkylated MSP4/5 is still able to afford protection (17). Furthermore, in rodent malaria challenge studies, $MSP1_{19}$ (28) and AMA1 (10) did not protect from heterologous challenge in their recombinant forms. There were differences in 17 out of 96 (17.7%) and 36 out of 567 (6.4%) amino acids, respectively, between the immunizing antigen and the challenge strain. For $MSP1_{19}$, this difference is much greater than the 8 amino acids out of 120 (6.7%) known to be polymorphic to a limited extent for *P. falciparum* (14, 15, 27, 32). However for AMA1, this difference is only slightly greater than the largest difference between the products of two alleles for *P. falciparum*, which is 32 out of 622 amino acids (5.1%) (23).

Antibody cross-reactivity for *P. y. yoelii* YM, *P. berghei* ANKA, and *P. c. adami* DS MSP4/5 is reduction sensitive, and for *P. berghei* ANKA MSP4/5, so too is protection. The EGF-like domain is the only structure predicted to be reduction sensitive from sequence analysis, and when compared across the three species, this domain differs at only 6 amino acids out of 37 (16%) (16). Given this level of conservation, and the reduction-sensitive nature of cross-reactivity, it is perhaps surprising that *P. berghei* ANKA MSP4/5 confers protection against *P. y. yoelii* YM challenge whereas *P. c. adami* DS MSP4/5 does not. Previous reduction and alkylation experiments using *P. y. yoelii* MSP4/5 have found that homologous protection is not dependent on the conformation of the EGF-like domain of MSP4/5, although peak parasitemias were elevated (17). It could be argued that the difference between the parasite burdens in mice in the reduced and nonreduced recombinant protein preparations equates to the protection afforded by reduction-sensitive conformation; however, there is no difference in survival outcome. Perhaps the antibody responses can be divided into two groups, those directed against reduction-insensitive linear epitopes and those directed against reduction-sensitive epitopes, and both types of response alone are capable of affording some degree of homologous protec-

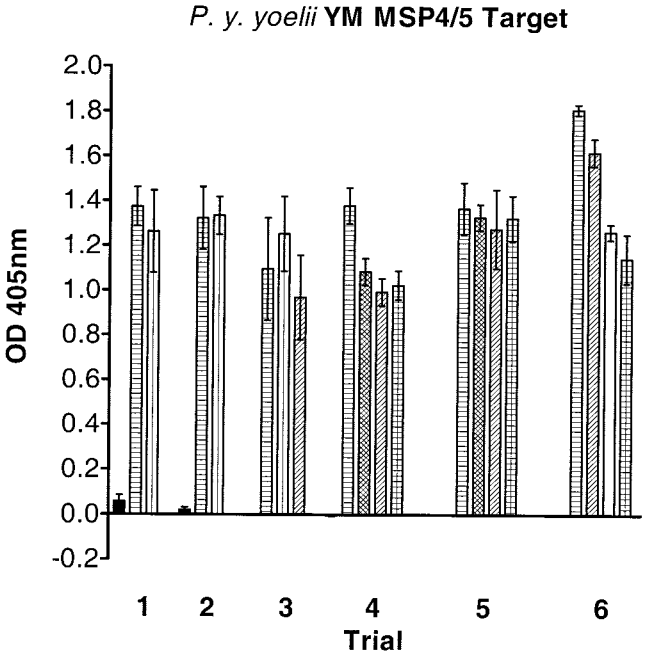


FIG. 4. Prechallenge antibody levels of immunized mice. Mice were immunized with PBS (adjuvant control; solid bars), recombinant *E. coli*-derived MSP4/5-His from *P. y. yoelii* YM (horizontally hatched bars), *P. y. killicki* 193L (vertically hatched bars), *P. y. nigeriensis* N67 (cross-hatched bars), *P. berghei* ANKA (diagonally hatched bars), reduced and alkylated *P. berghei* ANKA (open bars), and *P. c. adami* DS (checkered bars). The sera were diluted 1:5,000 in duplicate, and antibody levels were determined by ELISA on *E. coli*-derived *P. y. yoelii* YM MSP4/5. The mean optical density (OD) values at 405 nm are indicated, and the error bars represent standard errors of the mean. There were three, four, and five groups of mice in trials 1 and 2, trial 3, and trials 4, 5, and 6, respectively.

tion. However the EGF-like domain alone, expressed cytoplasmically in *E. coli* as a glutathione-*S*-transferase fusion or expressed periplasmically as a maltose binding protein fusion, conferred almost no protection (data not shown). Indeed, it appears that the full-length molecule is required to confer protection.

For MSP4, reduction and alkylation appear to transmit a conformational change through the molecule that alters antibody binding at epitopes remote from the EGF-like domain (33). We hypothesize that the tertiary structure of MSP4/5 may depend upon a partially or completely folded EGF-like domain. As recombinant *P. y. yoelii* YM and *P. berghei* ANKA MSP4/5 provide significant protection but *P. c. adami* DS MSP4/5 does not, comparing the primary amino acid sequences may highlight important protective N-terminal epitopes whose conformations, and perhaps functions, depend on an intact EGF-like domain. This type of analysis identifies blocks of similarity throughout the N terminus of MSP4/5 which would be useful to compare with structural data. Other considerations are that *P. c. adami* MSP4/5 may be too divergent to provide significant protection or may provide protection only if high antibody levels are achieved. Indeed, the four mice immunized with *P. c. adami* MSP4/5 that survived challenge had statistically significantly higher prechallenge antibody responses.

It was anticipated that differences in the primary amino acid sequence would result in decreased antibody binding and affinity, and consequently decreased protective immunity. Although no clear hierarchy in cross-reactivity related to the primary amino acid sequence was identified for nonreduced protein, the protective efficacy of the recombinant proteins is consistent with the hierarchy of polymorphism. The nature of the reduction-sensitive conformation, as well as the affinities and fine specificities of antibodies raised to these regions, has not been investigated and may provide an explanation for the different outcomes in *P. berghei* ANKA and *P. c. adami* DS MSP4/5-immunized mice, as may exploring the role of T-cell immunity in conferring protection.

Previous studies have found that mice immunized with whole-parasite preparations were afforded various degrees of heterologous protection within the rodent malaria parasite pairs *Plasmodium vinckei-P. chabaudi* and *P. berghei-P. yoelii*, and to a lesser extent between these two groups (8, 9, 24). Although different mechanisms of immunity (for example, cellular versus antibody) are implicated as being important in clearing *P. yoelii* and *P. chabaudi* infections and may in some cases have a component that is nonspecific in nature (6), the degree of protection loosely follows the phylogenic relatedness of the rodent malaria parasites (8, 9, 24). It is interesting that this study supports this finding.

This study demonstrates the robustness of a single recombinant antigen, MSP4/5, in conferring protection against a heterologous malaria challenge. There remains the question of its limits in terms of longevity of protection and how much the formulation can be improved, for example, by immunizing with antigen combinations (18), refolding (5), or using different adjuvants. The nature of the reduction-sensitive epitopes shared by *P. y. yoelii* YM, *P. berghei* ANKA, and *P. c. adami* DS MSP4/5 and how they relate to protection also need to be addressed. If such immunization regimes are to be adopted for *P. falciparum* MSP4 and MSP5, it must be in conjunction with adjuvants suitable for human use and capable of inducing an enduring immune response of sufficient magnitude and suitable type.

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