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Immunization with a Recombinant C-Terminal Fragment of *Plasmodium yoelii* Merozoite Surface Protein 1 Protects Mice against Homologous but Not Heterologous *P. yoelii* Sporozoite Challenge

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It has been reported previously that immunization with recombinant protein containing the two epidermal growth factor (EGF)-like modules from merozoite surface protein 1 (MSP-1) of *Plasmodium yoelii* (strain YM) protects mice against a lethal blood-stage challenge with the same parasite strain. Since MSP-1 is expressed in both liver- and blood-stage schizonts and on the surface of merozoites, we evaluated the effectiveness of immunization with recombinant proteins containing either the individual or the two combined EGF-like modules in producing a protective response against a sporozoite challenge. The recombinant protein expressing the combined EGF-like modules of the YM strain protected mice against a homologous sporozoite challenge, and sterile protection, as defined by the absence of detectable blood-stage parasites, was observed in the majority of the mice. In contrast, mice immunized with recombinant *P. yoelii* YM MSP-1 were not protected against a heterologous challenge with sporozoites from strain 265 BY of *P. yoelii*. The lack of protection may be explained by differences identified in the amino acid sequences of MSP-1 for the two strains. A recombinant protein containing the two EGF-like modules of MSP-1 from *P. yoelii* 265 BY was produced and used to immunize mice. These mice were protected against a homologous challenge with sporozoites of *P. yoelii* 265 BY. The results suggest that a recombinant MSP-1 has potential as a vaccine against malaria, but its efficacy may be limited by sequence polymorphism and selection of variants.

Malaria parasite infection is initiated by the bit of an infected mosquito, which injects sporozoites into the host's circulation. Sporozoites rapidly invade hepatocytes, where they differentiate to form schizonts that give rise to thousands of merozoites. These merozoites invade erythrocytes and initiate the asexual erythrocytic cycle that is responsible for the pathology of the disease. Liver- and blood-stage parasites share antigens (1, 37), and it has been postulated that immunity induced by antigens at one stage could affect other stages (29). For example, immunization with a recombinant malaria parasite heat shock protein (a protein that is expressed at all stages in the vertebrate host) influenced parasite development in the liver, leading to increased gametocyte numbers (31).

Merozoite surface protein 1 (MSP-1) is a well-characterized protein present in all species of *Plasmodium* (for reviews, see references 12 and 19). This precursor protein is synthesized during schizogony (18), and at the time of merozoite release and erythrocyte invasion, it is cleaved into several fragments. Only a 19-kDa C-terminal fragment (MSP-1_{19}), comprised of two epidermal growth factor (EGF)-like modules (4), remains bound to the merozoite membrane during invasion (3). Antibodies directed to this fragment have been shown to inhibit *Plasmodium falciparum* merozoite invasion of erythrocytes in vitro (3, 11) and by passive immunization to protect mice against a challenge of *Plasmodium yoelii* asexual blood-stage parasites (13, 26, 28). In vaccination experiments with recombinant polypeptides encompassing the 19-kDa region from *P. yoelii* MSP-1, immunized mice were protected against challenge with blood-stage parasites and protection was largely mediated by antibodies (10, 13, 14, 24, 25). Encouraged by these results and epidemiological data from The Gambia showing a correlation between anti-*P. falciparum* MSP-1_{19} antibody titer and reduced malaria morbidity (16, 38), further experiments were performed with monkeys. Conflicting results were observed. In one study, immunization with a recombinant yeast protein consisting of *P. falciparum* MSP-1_{19} fused to T-cell epitopes of tetanus toxin, together with Freund's adjuvant, protected *Aotus nancymai* but not *Aotus vociferans* against a lethal blood-stage challenge (22). The mechanism of protection seemed to be independent of antibodies. In a second study, *Aotus nancymai* monkeys were not protected by vaccination with a recombinant bacterial *P. falciparum* MSP-1_{19} in liposomes against a challenge with either homologous or heterologous *P. falciparum* blood-stage parasites (7). In summary, results obtained from both in vitro and in vivo experiments suggest that MSP-1_{19} is an important target of immunity against blood-stage malaria and may be an important antigen for inducing a protective response by vaccination. However, further insights into the nature of immune effector mechanisms are needed.

MSP-1 is also detected in liver schizonts of *P. falciparum* and *Plasmodium berghei* (1, 39, 40), and its presence is maximal when liver schizonts are fully mature (39). This suggests that the hepatic stage of parasite development can also be a target for immune responses induced by immunization with MSP-1. In the present work, we have studied the ability of immuniza-

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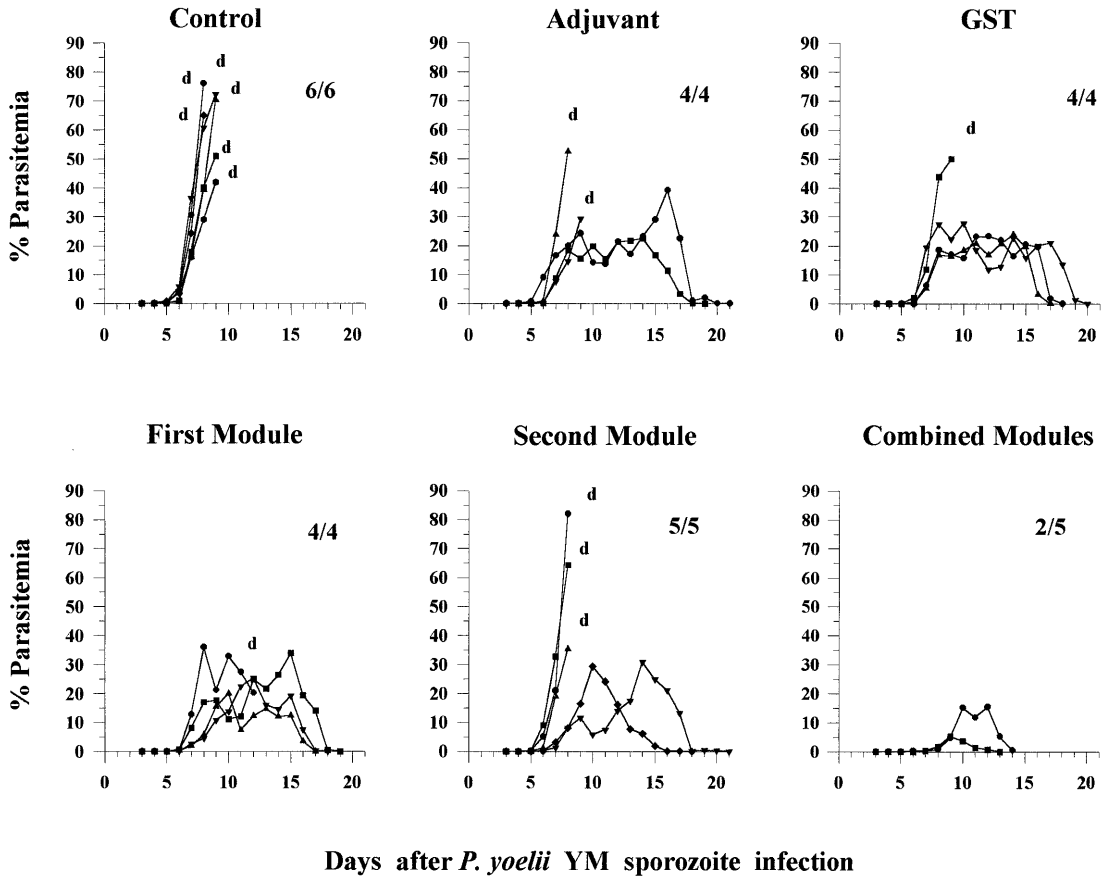

FIG. 1. Blood-stage parasitemia of immunized animals challenged with homologous *P. yoelii* YM sporozoites. Groups of BALB/c mice were either unimmunized (control) or immunized with Freund's adjuvant alone (adjuvant), with GST, or with various GST fusion proteins containing either the first EGF-like module, the second EGF-like module, or the two combined EGF-like modules from the C terminus of *P. yoelii* YM MSP-1, together with Freund's adjuvant. After i.v. challenge with 4,000 sporozoites, the parasitemia in each animal was monitored by examination of blood films stained with Giemsa reagent. Only points at which parasitemia was patent are plotted. In the top right-hand corner of each panel is shown the number of mice with patent parasitemia relative to the total number of mice in the group. d, dead mouse.

tion with recombinant MSP-1 to induce protective immunity in mice challenged with both homologous and heterologous *P. yoelii* sporozoites.

MATERIALS AND METHODS

Antigens. Recombinant proteins based on the C terminus of *P. yoelii* YM MSP-1 and encompassing either each individual or both EGF-like modules were expressed in *Escherichia coli* as fusion proteins with glutathione *S*-transferase (GST). The proteins were purified as described previously (24, 25).

The DNA coding for the C-terminal EGF-like modules of MSP-1 was amplified from *P. yoelii* 265 BY genomic DNA by the PCR, with primers based on the *P. yoelii* YM sequence and reaction conditions as described previously (24, 25). The amplified DNA was ligated into the TA cloning vector (Invitrogen), as described in the manufacturer's instructions, and clones containing inserts were identified. The inserts were excised from the TA vector with the restriction enzymes *Bam*HI and *Bgl*II and subcloned into the expression vector pGEX3X cut with *Bam*HI. Clones with inserts in the correct orientation were identified by PCR amplification and then sequenced with an Applied Biosystems 373A automatic DNA sequencer, as described in the manufacturer's instructions. The GST fusion proteins were expressed and purified by the methods described previously (24, 25).

Immunization schedule. Groups of 5- to 8-week-old female BALB/c mice purchased from Charles River (Saint Aubin-lès-Elbeuf, France) were immunized at the base of the tail with 30 μg of recombinant protein emulsified in complete Freund's adjuvant (Sigma Chemical Co., St. Louis, Mo.). A boost with 30 μg of the same protein emulsified in incomplete Freund's adjuvant (Sigma) was given 2 weeks and 3 weeks after the primary immunization. Groups of control mice received GST with adjuvant or the adjuvant alone.

Parasites. Sporozoites of the YM or 265 BY strain of *P. yoelii* were obtained from infected salivary glands of *Anopheles stephensi*. After aseptic dissection, the salivary glands were homogenized with a glass grinder and suspended in phosphate-buffered saline. One week after the last boost, mice were challenged intravenously (i.v.) with 4,000 sporozoites. Blood-stage parasitemia was assessed daily by examination of blood smears stained with Giemsa reagent from day 2 after challenge.

Antibody assay. Prechallenge antibody titers were assessed by an indirect immunofluorescence assay (IFA) as described previously (42), with slides coated with the blood of mice infected with each *P. yoelii* strain. Titers were expressed as the reciprocal of the highest serum dilution giving a positive fluorescence reaction.

RESULTS AND DISCUSSION

To determine whether immunity induced by immunization was effective against a sporozoite challenge, mice were immunized with recombinant proteins containing either the individual EGF-like modules or the combined two EGF-like modules of *P. yoelii* YM MSP-1 and challenged with 4,000 sporozoites from strain YM. Only immunization with the recombinant protein containing the two EGF-like modules gave protection; in this group, three of five mice did not develop patent blood-stage parasitemia, and the two remaining mice displayed only a transient parasitemia (Fig. 1). Interestingly, immunization with Freund's adjuvant alone transformed the normally lethal infection induced by *P. yoelii* YM sporozoites into a nonlethal infection. This is different from that observed previously when mice immunized with Freund's adjuvant and carrier protein were challenged directly with blood-stage parasites (22). This may be explained by a nonspecific activation of the immune system, leading to release of lymphokines and toxic mediators (2, 34, 35, 43) known to have an effect on the liver stage of the

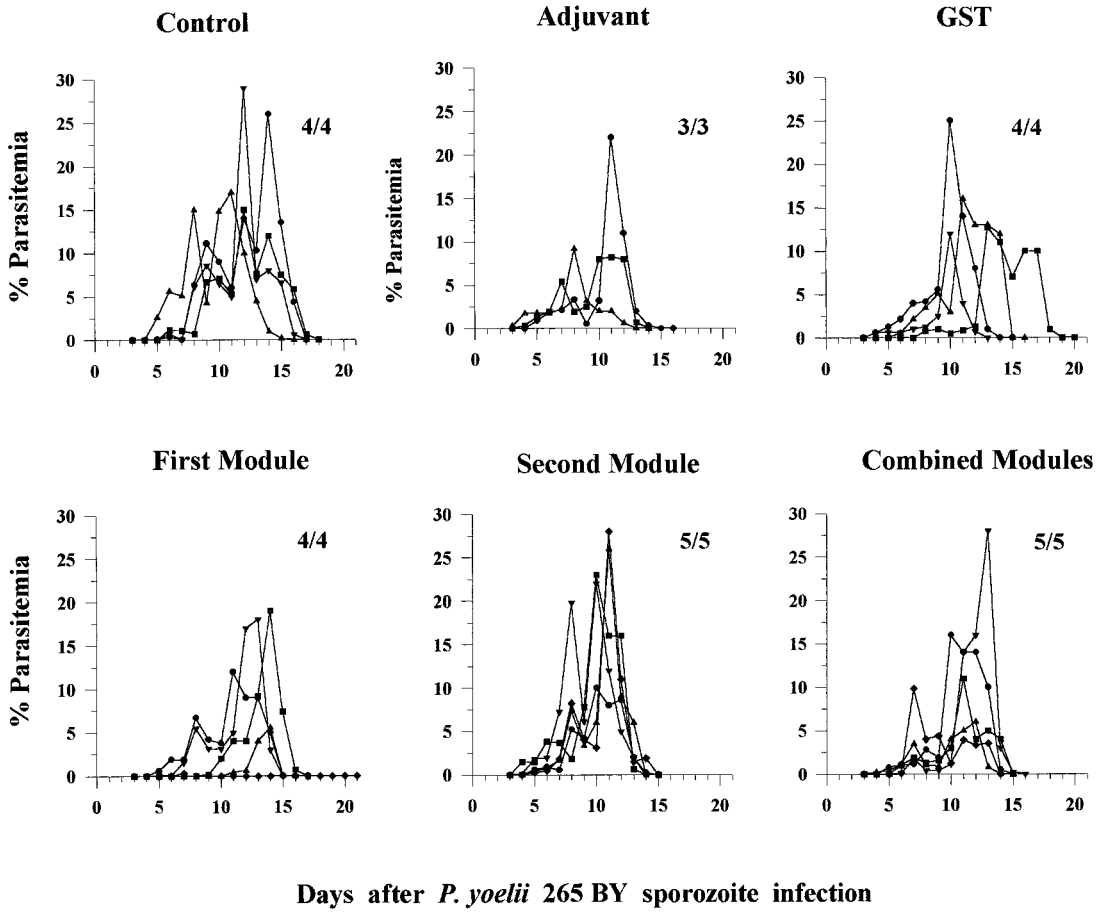

FIG. 2. Blood-stage parasitemia of immunized animals challenged with heterologous *P. yoelii* 265 BY sporozoites. Groups of BALB/c mice were immunized as described in the legend to Fig. 1 with recombinant proteins based on *P. yoelii* YM MSP-1 in Freund's adjuvant. After challenge, the blood-stage parasitemia was monitored. In the top right-hand corner of each panel is shown the number of mice with patent parasitemia relative to the total number of mice in the group.

malaria parasite (17, 30, 32–34). Of note, we have previously shown that injection of certain adjuvants [poly(AU) or muramyl peptide] induces nitric oxide synthesis in hepatocytes in vivo, leading to partial inhibition of malaria parasite liver-stage development (34).

We next asked whether the protective immunity induced was strain specific. Mice immunized with the various recombinant proteins based on strain YM MSP-1 were challenged with 4,000 heterologous sporozoites of strain 265 BY. Protection against challenge with 265 BY sporozoites was not observed in any of the groups of mice (Fig. 2).

Serum samples from mice immunized with the various constructs, and collected before challenge infection, were pooled and tested by IFA on blood-stage parasites. High antibody titers against the homologous YM parasite were detected in the pooled sera from mice immunized with the recombinant proteins containing either the first EGF-like module or the two combined EGF-like modules, with the latter group containing the highest activity (Table 1). When tested on the heterologous 265 BY blood-stage parasites, the pooled sera from each group of mice immunized with the various recombinant proteins contained only low levels of cross-reactive antibodies.

Because of the inability of mice immunized with the recombinant proteins containing the MSP-1 amino acid sequence of the YM strain to resist a challenge with *P. yoelii* 265 BY sporozoites, we compared the sequence of the corresponding region of MSP-1 from strain 265 BY with that of strain YM (23). The DNA coding for the C-terminal region of MSP-1 was amplified from *P. yoelii* 265 BY genomic DNA by the PCR and sequenced after cloning into plasmid pGEX3X. The inserts in two independent clones were sequenced and found to be identical. The deduced amino acid sequences from both strains were compared (Fig. 3). Within the 96-amino-acid region amplified from 265 BY MSP-1 (excluding the sequence coded by the oligonucleotide primers), there are 17 amino acid differences from the YM sequence, including one deletion and one insertion. Of these differences, 5 are located in the first EGF-

TABLE 1. Antibody response of mice immunized with various GST fusion proteins[a]

Immunogen	IFA titer[b]	
	YM	265 BY
None	—	—
Freund's adjuvant	—	—
First EGF-like module	25,600	400
Second EGF-like module	800	400
Combined EGF-like modules	102,400	400

[a] Antibody responses were based on the C terminus of *P. yoelii* YM MSP-1 and measured by immunofluorescence against blood-stage parasites of both YM and 265 BY strains.

[b] IFA titers correspond to the highest serum dilutions producing positive immunofluorescence. —, absence of any reactivity in the IFA.

First EGF-like module:

```
265 BY: MDGMDLLGVD PKHVCVDTRD IPKNAGCFRY DNGNEEWRCL LGFKK-ENNT CVEDNNPS
                                      *   *           *  *  *     *   *
YM:     MDGMDLLGVD PKHVCVDTRD IPKNAGCFRD DNGTEEWRCL LGYKKGEGNT CVENNNPT
              ↑
```

Second EGF-like module:

```
265 BY: CDTNNGGCDT AASCQTGDRS GENSKKVICT CKEPTPNAYY EGVFCSSSS
          *      * *    ***** *     *
YM:     CDINNGGCDP TASCQNAEST -ENSKKIICT CKEPTPNAYY EGVFCSSSS
```

FIG. 3. A comparison of the deduced amino acid sequences of the C-terminal regions of MSP-1 from *P. yoelii* 265 BY and YM. In the first module, the second and fourth cysteines found in most EGF-like structures are not present. The putative N-terminal amino acid at the cleavage site for the enzyme involved in MSP-1 processing is indicated (↑). The region linking the two modules is comprised of seven amino acids, shown at the end of the first module. The underlined sequence is coded by the oligonucleotides used to amplify the genomic DNA. Differences between the two sequences are indicated (⋆).

like module (the 44 amino acids between the putative protease cleavage site and the fourth cysteine residue), 2 are in the 7-amino-acid linking region between the two modules, and 10 are in the 45 amino acids forming the second EGF-like module. Within the second module, there is a stretch of six amino acids between the third and fourth cysteines which is quite different between the two forms. In studies to map the epitope recognized by the protective monoclonal antibody 302 in the YM MSP-1, the sequence Cys-Val-Glu-Asn in the linking region between the two modules was identified as critical for monoclonal antibody binding (9). In natural variants in which the Asn is replaced by Asp, antibody binding was abolished and the antibody was no longer effective on passive immunization (8). Interestingly, the 265 BY sequence falls into this category, since it has the Asp residue.

To determine whether mice immunized with the two EGF-like modules of *P. yoelii* 265 BY MSP-1 could be protected against a homologous challenge with 265 BY sporozoites, the fusion protein with GST was used to immunize a group of BALB/c mice. When these mice were challenged with 4,000 sporozoites of *P. yoelii* 265 BY, three of five animals had no patent blood-stage parasitemia and the other two had a patent parasitemia of shorter duration compared to that of controls. In the control groups, the typical self-limiting course of infection with this parasite was observed (Fig. 4).

A pool of serum samples from mice immunized with the 265 BY recombinant protein had a high antibody titer (102,400) by IFA against the homologous blood-stage parasite, but with the heterologous YM blood-stage parasite, only a low level of cross-reactive antibodies (titer, 800) was observed.

These results demonstrate that immunization with a recombinant protein containing the two EGF-like modules of *P. yoelii* MSP-1 induce protective immunity against challenge with sporozoites of the homologous strain. Protection was associated with a high level of antibodies recognizing asexual blood-stage parasites. The antibodies also recognized liver-stage schizonts (data not shown). Since fully protected mice had no patent blood-stage parasitemia, the targets for the protective responses could be hepatocytes containing mature schizonts, merozoites released from liver schizonts, and/or blood-stage parasites; the experiments described here do not discriminate between these possibilities. It is possible that antibodies to MSP-1 may act by inhibiting the proteolytic processing of MSP-1 (5), by agglutinating newly emerging merozoites (25), or by other mechanisms such as antibody-dependent cellular inhibition (6). In addition, immunization with MSP-1_{19} may induce T cells able to eliminate parasitized cells either from the liver or from the blood. Previous work showed that immunization with the individual EGF-like modules alone was not effective against blood-stage challenge (9, 25); the results reported here do not suggest that a more effective response against sporozoite challenge is induced. Experiments are in progress to determine what mechanisms are involved in protection against sporozoite challenge.

One important finding is that amino acid sequence differences limited protection against heterologous challenge. A comparison of the sequence reported here and that of others (15, 23) suggests that the EGF-like modules in *P. yoelii* MSP-1 are much more variable than the corresponding region of *P. falciparum* MSP-1, in which only four amino acids have been found consistently to vary in a limited way (20, 21, 41). One way to provide protection against all strains would be to use a vaccine containing all the possible sequence variants. Longitudinal studies have shown a correlation between serological responses to the C-terminal region of *P. falciparum* MSP-1, resistance to subsequent malaria infection (38), and clinical immunity (16). Naturally acquired immunity may result in part from the progressive addition of protective immune responses to the different MSP-1 variants present in a particular location. Further studies are required to design an effective vaccine based on the MSP-1 molecule.

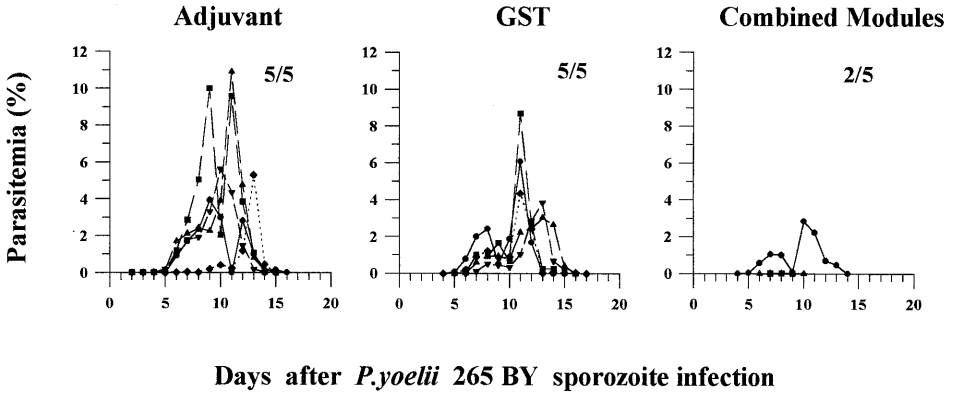

FIG. 4. Blood-stage parasitemia of immunized animals challenged with homologous *P. yoelii* 265 BY sporozoites. Groups of BALB/c mice were immunized with Freund's adjuvant alone (adjuvant) or with GST or a GST fusion protein containing the two combined EGF-like modules from the C terminus of *P. yoelii* 265 BY MSP-1, together with Freund's adjuvant. After i.v. challenge with 4,000 sporozoites, the parasitemia in each animal was monitored by examination of blood films stained with Giemsa reagent. Only points at which parasitemia was patent are plotted. In the top right-hand corner of each panel is shown the number of mice with patent parasitemia relative to the total number of mice in the group.

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