Analysis of Immunological Nonresponsiveness to the 19-Kilodalton Fragment of Merozoite Surface Protein 1 of *Plasmodium yoelii*: Rescue by Chemical Conjugation to Diphtheria Toxoid (DT) and Enhancement of Immunogenicity by Prior DT Vaccination

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The *Plasmodium* merozoite surface protein 1 (MSP1) is a leading vaccine candidate for protecting against the blood stage of malaria. Previous studies have shown that the 19-kDa carboxyl terminus of this protein is able to induce protective immunity in some monkey and mouse strains. We show that immunization with the recombinant *Plasmodium yoelii* 19-kDa fragment of MSP1 (MSP1₁₉) expressed in *Saccharomyces cerevisiae* (yMSP1₁₉) can induce protective antibodies in several inbred mouse strains and one outbred mouse strain. However, mice expressing the *H-2^s* major histocompatibility complex haplotype are unable to generate yMSP1₁₉-specific antibodies. While synthetic peptides derived from MSP1₁₉ are immunogenic in B10.S mice, they cannot function as helper epitopes, and immunization with yMSP1₁₉ does not induce T cells that recognize the recombinant protein or synthetic peptides corresponding to its sequence. Nonresponsiveness could be overcome by using chemical linkers to conjugate yMSP1₁₉ to diphtheria toxoid (DT), resulting in immunogens capable of inducing protective yMSP1₁₉-specific antibodies in both MSP1₁₉-responsive and otherwise nonresponsive mouse strains. The ability of sera from mice immunized with the conjugate to inhibit binding of a protective monoclonal antibody (MAb 302) to yMSP1₁₉ correlated strongly with a delay in the prepatent period. Chemical conjugation of yMSP1₁₉ to DT may be a preferred method to enhance immunogenicity, as carrier priming experiments demonstrated that an existing immune response to DT enhanced a subsequent antibody response to yMSP1₁₉ after vaccination with yMSP1₁₉-DT. These results have important implications for the development of a malaria vaccine to protect a population with diverse HLAs.

Malaria remains one of the leading causes of morbidity and mortality in the tropics. Each year, 300 to 500 million cases of malaria occur, and 1 to 2 million of these cases result in death (90% of these deaths occur in Africa) (33). Existing control measures, such as chemoprophylaxis, are increasingly less efficacious, emphasizing the need to develop a successful vaccine against the disease.

Merozoite surface protein 1 (MSP1) is a leading vaccine candidate against the blood stage of malaria and has been evaluated extensively in rodent and primate models (7, 12, 13, 18, 19). It is expressed on the surface of the developing merozoite where it undergoes two proteolytic cleavages, the second of which generates a 19-kDa fragment (MSP1₁₉) that remains membrane bound and is carried on the surface of the merozoite into the newly invaded erythrocyte (2, 3). MSP1₁₉ is cysteine rich and highly conserved and contains two epidermal growth factor (EGF)-like domains (4). It is the first EGF-like domain in the 19-kDa fragment in *Plasmodium yoelii* that is the target of an immunoglobulin G3 (IgG3) protective monoclonal

antibody (MAb 302) (5). MSP1₁₉ has been produced using a number of recombinant protein expression systems, including bacterial (7), mammalian (22), baculovirus (6), and yeast (15) models, which have all demonstrated some degree of success at producing antigens that are both immunogenic and protective against challenge with the malaria parasite. Immunity induced by MSP1₁₉ is thought to be dependent on a high antibody titer at the time of challenge (12, 13) and on an ongoing immune response induced by the malaria parasite following challenge (14), the specificity of which need not be directed at MSP1₁₉ (32).

Despite extensive investigation, a significant obstacle to the ultimate success of MSP1₁₉ as a vaccine is its small size, which may make it nonimmunogenic in a significant percentage of the population. Immunization with glutathione *S*-transferase (GST)-MSP1₁₉ expressed in *Escherichia coli* can protect some but not all *H-2* congenic strains of mice following challenge with *P. yoelii* (31). Protection correlated with the genes present in the *H-2* loci. Further studies found that immunization with MSP1₁₉ expressed in *Saccharomyces cerevisiae* (yMSP1₁₉), which lacked the GST molecule but contained six additional histidine residues, resulted in sterile and complete protection from challenge in two *H-2* congenic mouse strains following either parental or intranasal immunization (12–14).

The problem of designing a vaccine that is universally recognized by a population with diverse HLAs is a challenge for

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FIG. 1. The course of parasitemia in different strains of mice immunized with γ MSP1₁₉ (closed symbols) and PBS (open symbols) in FA and subsequently challenged with *P. yoelii* YM. Mice were immunized on day 0 with 20 μ g of γ MSP1₁₉ in CFA and then given booster doses of 20 μ g of γ MSP1₁₉ in IFA on days 21, 42, and 56. A final booster dose of 20 μ g of γ MSP1₁₉ in PBS was given on day 63. Mice were challenged i.v. on day 70 with 10^4 *P. yoelii* YM parasitized red blood cells. Symbols depict parasitemia in individual mice.

malaria vaccine development. A number of studies have focused on the use of universal helper T-cell epitopes to provide help for B cells, thereby enhancing the immunogenicity of small-subunit-based vaccines (1, 17, 24). While conjugating B-cell epitopes (haptens) to proteins is a more classical approach to providing T-cell help (21) and one that would result in immunological responsiveness among a greater proportion of the population, some studies have suggested that prior exposure to the protein can result in a diminished response to the hapten following protein-hapten immunization (8, 11, 23, 26–

28). However, this is not necessarily observed (8, 23, 29), and furthermore, protein-protein conjugates have not been studied extensively. If prior exposure to a protein vaccine (such as diphtheria toxoid [DT]) resulted in enhanced immunogenicity following subsequent immunization with a DT-protein vaccine, then this would be an additional strategy to develop a vaccine that is highly immunogenic in a large proportion of the population.

We defined genetic restriction of the immune response to γ MSP1₁₉ in a number of inbred strains and one outbred strain

TABLE 1. Sequences of the 10 overlapping linear peptides derived from yMSP1₁₉

Peptide	Sequence
15.....	NMDGMDLLGVDPKHVCVDTR
16.....	DPKHVCVDTRDIPKNAGCFF
17.....	DIPKNAGCFRDDNGTEEWRC
18.....	DDNGTEEWRCLLGYKKGEGN
19.....	LLGYKKGEGNTCVENNNPTC
20.....	TCVENNNPTCDINNGGCDPT
21.....	DINNGGCDPTASCQNAESTE
22.....	ASCQNAESTENSKIICTCK
23.....	NSKKIICTCKEPTPNAYYEG
24.....	EPTPNAYYEGVFCSSSS

of mice immunized with yMSP1₁₉. An inbred strain of mice that failed to generate any antibodies to yMSP1₁₉ was found to be responsive to two different DT conjugates and was protected from challenge. Furthermore, prior exposure to DT enhanced the subsequent immune response to yMSP1₁₉, when it was administered as a DT conjugate.

MATERIALS AND METHODS

Mice and parasite. Female BALB/c (*H-2^d*), C57BL/10 (*H-2^b*), B10.BR (*H-2^k*), B10.D2 (*H-2^d*), and Quackenbush mice were 6 to 10 weeks old at the start of the experiments. Mice were obtained from the Animal Resources Centre, Willetton, Australia. Female B10.S (*H-2^s*) mice were 10 to 15 weeks old at the start of experiments and were obtained from the Walter and Eliza Hall Institute, Melbourne, Australia. All mice were housed under specific-pathogen-free conditions. The experimental procedures described in this paper were approved by The Bancroft Centre Animal Ethics Committee.

The malaria parasite used was *P. yoelii* YM (lethal). The parasites were maintained by intraperitoneal passaging of 10⁶ parasitized erythrocytes into recipient mice. The parasitized red blood cells were cryopreserved in liquid nitrogen.

yMSP1₁₉ protein and peptides. Recombinant MSP1₁₉ corresponding to *P. yoelii* YM protein was produced with a C-terminal His₆ tag in *S. cerevisiae* (31). It was provided as a generous gift by Anthony Stowers (National Institute of Allergy and Infectious Diseases, National Institutes of Health).

Ten peptides, 20 amino acids in length spanning the length of MSP1₁₉ and overlapping each other by 10 amino acids, were made by the Peptide Unit, Queensland Institute of Medical Research and Mimotopes Pty., Ltd., Victoria, Australia (30). The peptide sequences are listed in Table 1. The purity of the peptides was >85%.

Preparation of yMSP1₁₉-DT conjugate using SPDP. First, yMSP1₁₉ and DT were mixed individually with *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP). The *N*-succinimidyl moiety of SPDP coupled with the primary amine of yMSP1₁₉ and DT to form a stable amide bond. The resulting DT 2-pyridyl disulfide derivative was subsequently reduced to give DT molecules with free thiol groups. Finally, the 2-pyridyl disulfide-containing yMSP1₁₉ was mixed with the thiol-containing DT, yielding a yMSP1₁₉-DT conjugate linked via a disulfide bond. The conjugate was dialyzed against phosphate-buffered saline (PBS) overnight at 4°C to eliminate free yMSP1₁₉. This conjugate was named yMSP1₁₉-DT (SPDP). Determination of conjugate concentration was determined with a Bradford protein assay kit (Bio-Rad).

Preparation of yMSP1₁₉-DT conjugate using 6-maleimidocaproic acyl *N*-hydroxysuccinimide ester (MCS). DT was dialyzed against a solution consisting of 50 mM triethanolamine, 0.15 M NaCl, and 1 mM EDTA (pH 8.0). A 10× molar excess of 2-iminothiolamine-HCl (Traut's reagent; Pierce) was added to DT prior to a 45-min incubation under N₂ at room temperature (RT). The Traut's reagent reacted with the primary amines on DT, introducing sulfhydryl functional groups. Spare Traut's reagent was separated from the iminothiolated protein by dialysis against PBS.

Initially, the conjugation process was optimized to determine the amount of chemical linker to add to the reaction mixture. Consequently, yMSP1₁₉ suspended in phosphate buffer was added to a 2× molar excess of MCS (Sigma) dissolved in dimethyl formamide and allowed to react for 1 h at RT. The *N*-hydroxysuccinimide moieties of MCS coupled with the primary amines of

yMSP1₁₉ to form a stable amide bond. The MCS-yMSP1₁₉ solution was dialyzed overnight at 4°C against a solution containing 0.1 M phosphate and EDTA (pH 7.0).

The iminothiolated DT was added to MCS-yMSP1₁₉ in a 2× molar excess and was allowed to react for 1 h at RT. The free maleimide group of MCS reacted with the sulfhydryl group of the iminothiolated DT, forming a stable thioether linkage. This yMSP1₁₉-DT conjugate was then dialyzed against PBS overnight at 4°C to eliminate free yMSP1₁₉. The conjugate was named yMSP1₁₉-DT (MCS). The conjugate concentration was determined with a Bradford protein assay kit (Bio-Rad).

Analysis of yMSP1₁₉-DT conjugate. To ensure yMSP1₁₉ had conjugated to DT, the products were run on a sodium dodecyl sulfate–polyacrylamide gel (4 to 20% polyacrylamide) (Gradipore). The protein bands were visualized by staining with Coomassie blue and by Western blotting. The yMSP1₁₉-DT conjugate on the Western blot was detected by either MAb 302 (20), polyclonal yMSP1₁₉ hyperimmune serum (HIS) or DT HIS raised in mice. A horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was used as the secondary antibody.

yMSP1₁₉ and yMSP1₁₉ conjugate protection studies. For the yMSP1₁₉ protection studies, groups of five mice were immunized subcutaneously (s.c.) with 20 µg of yMSP1₁₉ in complete Freund's adjuvant (CFA). At day 0, equal amounts of emulsion (10 µg at each site) were injected into the base of the tail and abdomen. The mice were given booster doses. On day 21, a booster dose of 20 µg of yMSP1₁₉ in incomplete Freund's adjuvant (IFA) was injected s.c. at the base of the neck. On days 42 and 56, a booster dose of 20 µg of yMSP1₁₉ in IFA was injected intraperitoneally (i.p.). The mice were given a final booster dose i.p. on day 63 with the same amount of yMSP1₁₉ in PBS.

Mice were challenged intravenously (i.v.) with 10⁴ *P. yoelii* YM parasitized erythrocytes on day 70 of the immunization protocol. Parasitemia was monitored by microscopic examination of stained blood films every second day following challenge infection. Blood was collected by snipping the tail prior to each booster dose and every second day following challenge infection for the duration of the experiment and frozen until it was assayed for antibody levels.

Immunization studies with the yMSP1₁₉-DT conjugates [named yMSP1₁₉-DT (MCS) and yMSP1₁₉-DT (SPDP)] followed the same schedule and dosage as described above for yMSP1₁₉, except that the mice received two additional booster doses in PBS administered i.p. on days 114 and 120. (The two extra immunizations helped to increase the yMSP1₁₉-specific antibody levels). In the yMSP1₁₉-DT conjugate experiment, the mice that were immunized with yMSP1₁₉ alone also received the two additional booster doses. The mice received 20 µg of protein in each immunization and were challenged with 10⁴ *P. yoelii* YM parasitized erythrocytes 7 days (day 127) after the final booster dose.

Determination of the effect of prior exposure to DT. Groups of mice (C57BL/10 and B10.S mice; five mice per group) were immunized s.c. at the base of the tail and abdomen with 20 µg of yMSP1₁₉, yMSP1₁₉-DT (SPDP), DT, or PBS in CFA (10 µg at each site). Twenty-one days later, the mice were given s.c. booster doses of 20 µg of yMSP1₁₉-DT (SPDP) in IFA at the base of the neck. Blood samples were collected for the duration of the experiment, and serum samples were obtained for determination of DT- and yMSP1₁₉-specific antibody responses.

Priming mice with MSP1₁₉ or its peptides. The mice were injected s.c. at two sites with 20 µg of MSP1₁₉, 20 µg of peptide, or PBS emulsified in CFA. Three weeks later, the mice were given s.c. booster doses of 20 µg of yMSP1₁₉ emulsified in IFA. Blood was collected from the tail tip immediately prior to the booster dose and every 2 days for the length of the experiments. Serum was frozen until assayed for antibody levels.

FIG. 2. yMSP1₁₉-specific antibody titers as measured by ELISA for mice immunized with yMSP1₁₉ (closed symbols) and PBS (open symbols) in FA and subsequently challenged with *P. yoelii* YM. Mice were immunized on day 0 with 20 µg of yMSP1₁₉ in CFA and then given booster doses of 20 µg of yMSP1₁₉ in IFA on days 21, 42, and 56. A final booster dose of 20 µg of yMSP1₁₉ in PBS was given on day 63. Mice were challenged i.v. on day 70 with 10⁴ *P. yoelii* parasitized red blood cells (indicated by arrows). Day 70 on these graphs is equivalent to day 0 in Fig. 1. Symbols depict antibody titer in individual mice.

Lymphocyte proliferation assays. Groups of 10 mice were immunized in the hind footpads with pools of five sequential overlapping peptides emulsified in CFA. They received 20 µg of each peptide in the immunization, with a cumulative total of 100 µg of peptide in each immunization. Seven to nine days later, the draining popliteal and inguinal lymph nodes were removed. Cells were suspended at 2.5×10^6 cells/ml in Eagle minimum essential medium containing 2-mercaptoethanol (diluted 1:1,000) and 2% normal mouse serum, and 200 µl of this suspension was added to 96-well plates. Cells were cultured in triplicate with various concentrations of peptides, antigens, or mitogens for 72 h at 37°C in 5% CO₂. The plates were pulsed with [³H]thymidine (Amersham Pharmacia), and incorporation of radiolabel was measured 18 to 24 h later by β-emission spectroscopy.

Antibody assay. Serum antibody levels were assayed by enzyme-linked immunosorbent assays (ELISAs). The wells in 96-well plates were coated with 0.5 µg of yMSP1₁₉ per ml in coating buffer and incubated overnight at 4°C. The plates were then blocked with PBS containing 1% bovine serum albumin for 1 h at 37°C. Serum previously diluted 1:10 in PBS was added to the wells and serially diluted, and the plates were incubated for 1 h at 37°C. After the wells were washed with PBS containing 0.05% Tween 20, a 1:3,000 dilution of HRP-conjugated goat anti-mouse IgG (The Binding Site) was added and incubated for 1 h. Following further washing, substrate solution [2,2'-azinobis(3-ethylbenzothiazolinesulfonic acid) (ABTS); Sigma] was added. After 30 min, the optical density was determined at 405 nm. For antibody isotyping ELISAs, HRP-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 were used (The Binding Site).

FIG. 4. Antigens recognized by T cells from draining lymph nodes of Quackenbush mice immunized with pools of overlapping peptides derived from yMSP1₁₉. T-cell proliferative responses of mice immunized with peptide pool 1 (peptides 15 to 19 [P15 to P19]) (left) or with peptide pool 2 (peptides 20 to 24 [P20 to P24]) (right) are shown. The cells were plated in triplicate, and each symbol is the mean of an individual mouse's response to each peptide. The peptide and protein concentration used to stimulate the T cells was 30 μ g/ml. Cells were incubated with purified protein derivative (PPD), concanavalin A (CONA), and Eagle minimum essential medium as controls.

For the inhibition ELISAs, coating, blocking, and addition of diluted sera were done as described above. However, an extra step involving the incubation of MAb 302 (1 μ g/ml) for 1 h was performed after the serum had been washed from the plate. The MAb 302 was then washed from the plates prior to the addition of a 1:3,000 dilution of HRP-conjugated goat anti-mouse IgG3 (The Binding Site) to detect the level of MAb 302 binding to yMSP1₁₉.

Statistical analysis. Data from the yMSP1₁₉-DT experiments were analyzed using Spearman rank correlation, Kruskal-Wallis test, or Mann-Whitney test. Values for P of <0.05 in the Kruskal-Wallis or Mann-Whitney test were considered statistically significant. The total area under the curve was used as an indicator of total antibody produced.

RESULTS

Determination of antibody response and protection in different strains of mice following vaccination with yMSP1₁₉. To assess the roles of H -2 genes on the immunogenicity of yMSP1₁₉, five inbred mouse strains, C57BL/10 (H -2^b), BALB/c (H -2^d), B10.D2 (H -2^d), B10.BR (H -2^k), and B10.S (H -2^s), and one outbred strain (Quackenbush) were immunized with yMSP1₁₉. Following completion of the immunization schedule, mice were challenged with *P. yoelii* YM.

Survival was observed in all yMSP1₁₉-immunized groups tested, although the levels of protection (as determined by peak parasitemia) varied in the different mouse strains (Fig. 1). Control mice (immunized with PBS) and B10.S mice immunized with yMSP1₁₉ developed a high parasitemia. Parasites were not detected in BALB/c and C57BL/10 mice immunized with yMSP1₁₉ (Fig. 1). With the exception of B10.S mice, all

strains generated yMSP1₁₉-specific antibodies following immunization (Fig. 2).

Determination of T-cell proliferative responses for peptides derived from yMSP1₁₉. To determine the T-cell determinants on yMSP1₁₉, mice were immunized with pools of synthetic overlapping peptides spanning yMSP1₁₉ (Table 1). Peptide pool 1 contained peptides 15, 16, 17, 18, and 19, and peptide pool 2 contained peptides 20, 21, 22, 23, and 24. The draining lymph nodes were taken 7 to 9 days later to assess responsiveness.

As had been previously shown (30), C57BL/10 mice responded to peptides 19, 23, 24, and yMSP1₁₉, whereas BALB/c mice responded to peptides 18 and 24 (Fig. 3). We now show that B10.D2 mice, with the same H -2 haplotype as BALB/c mice, responded to peptide 24, but not peptide 18. We observed that B10.BR (H -2^k) mice responded to peptide 16, while B10.S (H -2^s) mice responded to peptides 22 and 23 (Fig. 3). C57BL/10 mice were the only inbred mouse strain able to respond to yMSP1₁₉ following peptide immunization. Quackenbush (outbred) mice had different responses, but each mouse recognized multiple peptides. The most frequent responses were to peptides 16, 17, 23, and 24 (Fig. 4). They were also able to respond to yMSP1₁₉.

As B10.S mice did not produce antibodies following yMSP1₁₉ immunization yet responded to peptides following immunization with peptide pool 2, they were also immunized with the recombinant protein to determine peptide and protein

FIG. 3. Antigens recognized by T cells from the draining lymph nodes of different strains of mice immunized with pools of overlapping peptides derived from yMSP1₁₉. T-cell proliferative responses of mice immunized with peptide pool 1 (peptides 15 to 19 [P15 to P19]) (left graphs) or with peptide pool 2 (peptides 20 to 24 [P20 to P24]) (right graphs) are shown. Cells were incubated with various concentrations (30, 10, or 3 μ g/ml) of peptide or with purified protein derivative (PPD), concanavalin A (CONA), and Eagle minimum essential medium as controls. Bars are the means plus 95% confidence intervals (error bars) for the 10 mice in each group.

FIG. 5. yMSP1₁₉-specific antibody responses in different strains of mice primed with the immunodominant peptide(s) specific for that mouse strain, yMSP1₁₉, or PBS and then given a booster dose of yMSP1₁₉ on day 0. Bars are the means $\pm$ 95% confidence intervals (error bars) for the five mice in each group. Peptides 16 (P16), 18 (P18), 19 (P19), 22 (P22), 23 (P23), and 24 (P24) were used to prime mice.

responses. Following protein immunization, T cells from B10.S mice did not respond to any of the peptides or yMSP1₁₉ (data not shown).

Induction of T-cell help by MSP1₁₉ epitopes. We then asked whether proliferative T-cell epitopes could activate helper T cells. Mice were primed with putative peptide epitopes, and 3 weeks later, the mice were given booster doses of yMSP1₁₉ using an established assay (10, 30). yMSP1₁₉-specific antibody responses were then measured to determine whether priming with the peptide resulted in a more rapid antibody response after the yMSP1₁₉ booster dose.

At the time of the yMSP1₁₉ booster dose, mice that had previously been immunized with yMSP1₁₉ or with one of several peptides had yMSP1₁₉-specific antibody that was detectable by ELISA (Fig. 5). BALB/c mice primed with p18 or p24 and C57BL/10 mice primed with p19 or p24 had an earlier and more rapid yMSP1₁₉ antibody response than those primed with PBS, in agreement with previous studies (30). However, in B10.S, B10.BR, and B10.D2 mice primed with proliferative peptide epitopes, the rate of development of the yMSP1₁₉ antibody response was similar to that in mice primed with PBS, indicating that the peptides did not contain T-helper epitopes.

FIG. 6. Western blot of yMSP1₁₉-DT conjugates probed with yMSP1₁₉-specific HIS. Lane 1, molecular weight markers; lane 2, DT; lane 3, yMSP1₁₉; lane 4, yMSP1₁₉-DT (MCS); lane 5, yMSP1₁₉-DT (SPDP).

Preparation and immunogenicity of recombinant protein-DT conjugate. The above data indicated that B10.S mice were absolute nonresponders, and there was no evidence that they recognized any helper T-cell epitopes on yMSP1₁₉. To attempt to induce a yMSP1₁₉-specific antibody response in these mice, yMSP1₁₉ was chemically conjugated to DT. Two different conjugation procedures utilizing two different chemical linkers were used, SPDP and MCS. (Their chemistry is outlined in Materials and Methods.) The results of Western blot analysis showed that both conjugates reacted with yMSP1₁₉ HIS (Fig. 6). These bands were also recognized by MAb 302 (a protective monoclonal antibody that recognizes a conformational epitope in the first EGF-like domain of MSP1₁₉ [20]) and DT HIS [data not shown]. The absence of a band at a molecular weight of 19,000 indicated that the mixture contained only conjugated yMSP1₁₉ (Fig. 6). Responder C57BL/10 mice and nonresponder B10.S mice were immunized with PBS, DT, yMSP1₁₉, yMSP1₁₉-DT (MCS), or yMSP1₁₉-DT (SPDP) and challenged with malaria parasites to assess the ability of the conjugates to elicit a protective antibody response.

C57BL/10 mice immunized with yMSP1₁₉ produced specific antibodies and were protected from lethal challenge, whereas the B10.S mice immunized with yMSP1₁₉ did not produce yMSP1₁₉-specific antibodies and all these mice succumbed to challenge infection (Fig. 7A). However, both strains of mice immunized with either yMSP1₁₉-DT (MCS) or yMSP1₁₉-DT (SPDP) produced yMSP1₁₉-specific antibodies and were protected (Fig. 7B and C). C57BL/10 mice immunized with yMSP1₁₉-DT (MCS) and yMSP1₁₉-DT (SPDP) developed higher yMSP1₁₉-specific antibody titers (mean maximum antibody titers of 1,024,000 and 1,024,000, respectively) than B10.S mice (mean maximum antibody titers of 358,400 and 384,000, respectively) and exhibited a higher degree of protection

(mean peak parasitemias of 7.2 and 4%, respectively) than B10.S mice (mean peak parasitemias of 14.8 and 20%, respectively) (Fig. 7B and C). Mice immunized with DT or PBS and the B10.S mice immunized with yMSP1₁₉ were not protected (Fig. 7D and E). Sera from mice immunized with yMSP1₁₉-DT (MCS) or yMSP1₁₉-DT (SPDP) recognized conjugated yMSP1₁₉ and DT (data not shown).

The yMSP1₁₉-specific antibody response in both strains of conjugate-immunized mice was predominantly IgG1 and IgG2b, irrespective of which conjugate was used for immunization (data not shown).

After the immunized mice were challenged, there was a significant delay ($P < 0.05$) in the prepatent period (Fig. 8A)—the best indicator of effective MSP1₁₉-specific antibody function (14). The mean prepatent period was longest in the C57BL/10 mice immunized with yMSP1₁₉ (Fig. 8A). C57BL/10 and B10.S mice immunized with yMSP1₁₉-DT (MCS) or yMSP1₁₉-DT (SPDP) had extended prepatent periods that were significantly different ($P < 0.05$) from the prepatent periods seen for the groups of mice immunized with PBS or DT and for B10.S mice immunized with yMSP1₁₉.

Fine specificity of the antibody response. MAb 302 binds to an epitope in the first EGF-like domain of MSP1₁₉ and confers protection when it is passively transferred to mice (20). Recognition of this epitope could be used as a predictive indicator of protection after challenge. Competition ELISAs were performed to determine whether serum samples from immunized mice recognized this epitope.

Sera from C57BL/10 mice immunized with yMSP1₁₉, yMSP1₁₉-DT (MCS), or yMSP1₁₉-DT (SPDP) were more effective at inhibiting the binding of MAb 302 to yMSP1₁₉ than the sera from yMSP1₁₉ conjugate-immunized B10.S mice (Fig. 8B). However, sera from B10.S mice immunized with yMSP1₁₉-DT (MCS) or yMSP1₁₉-DT (SPDP) could significantly inhibit the binding of MAb 302 ($P < 0.05$) compared with sera from C57BL/10 and B10.S mice immunized with DT or PBS and B10.S mice immunized with yMSP1₁₉ (Fig. 8B). Overall, the percent inhibition values for both mouse strains immunized with the conjugates and for C57BL/10 mice immunized with yMSP1₁₉ were significantly different ($P < 0.05$) from the values for the control groups immunized with DT or PBS and for B10.S mice immunized with yMSP1₁₉. In both C57BL/10 and B10.S mice, there was a significant positive correlation between the level of inhibition exhibited by the antibodies in the serum and the length of the prepatent period ($r = 0.721$ and 0.92 , respectively; $P < 0.01$ for both groups) (Fig. 9).

Determining the effect of an existing immune response to the carrier protein (DT) on a subsequent immune response to yMSP1₁₉. While DT conjugation can overcome immunological nonresponsiveness, we were concerned that epitopic suppression as a result of prior exposure to DT may compromise a potential DT-based conjugate (11, 27). We thus asked whether an existing immune response to DT could interfere with the development of an immune response to yMSP1₁₉ after immunization with a yMSP1₁₉-DT conjugate. C57BL/10 and B10.S mice were immunized s.c. with 20 μ g of yMSP1₁₉, yMSP1₁₉-DT (SPDP), DT, or PBS in CFA. Twenty-one days later, they were immunized s.c. with 20 μ g of yMSP1₁₉-DT

(SPDP) in IFA. Sera were tested for the production of yMSP1₁₉-specific antibodies on day 21 after immunization.

C57BL/10 and B10.S mice preimmunized with DT were able to produce yMSP1₁₉-specific antibodies after they were given booster doses of the conjugate (Fig. 10). Indeed, yMSP1₁₉-specific antibodies were detected earlier ($P < 0.01$ for both mouse strains) and more antibody was produced ($P < 0.01$ for both mouse strains) (Materials and Methods) in mice preimmunized with DT than in mice preimmunized with PBS, indicating that the presence of DT antibodies at the time of yMSP1₁₉-DT immunization did not interfere with the production of yMSP1₁₉-specific antibodies (Fig. 10) but instead enhanced it.

DISCUSSION

This study has demonstrated that it is possible to generate protective antibodies after immunization with yMSP1₁₉ in a number of inbred and outbred mouse strains with different *H-2* genes. The inability to produce antibodies specific for yMSP1₁₉ after immunization would appear to be controlled by I-A^s, given that *H-2^s* mice do not express an I-E molecule (Fig. 2). This immunological nonresponsiveness was related to an absence of T helper epitopes on yMSP1₁₉ recognized by B10.S mice and could be overcome by chemical modification of yMSP1₁₉ with a known source of T helper epitopes, DT. Furthermore, DT preimmunization markedly enhanced the response to the conjugate.

In a previous study using GST-MSP1₁₉, *H-2^k* mice were not protected after immunization (31). They did not examine the ability of *H-2^s* mice to respond to immunization with GST-MSP1₁₉. Interestingly, a different pattern of nonresponsiveness was observed in the experiment presented here, with *H-2^k* mice exhibiting protection and *H-2^s* mice failing to generate antibodies when immunized with yMSP1₁₉ alone. There are a number of possible explanations for the differences observed for recombinant proteins from the different expression systems. The yMSP1₁₉ was expressed in *S. cerevisiae*, as opposed to GST-MSP1₁₉, which is produced in *E. coli*. It is likely that the presence of a hexahistidine tail at the C-terminal end of the yMSP1₁₉ molecule created an epitope not present in the GST-MSP1₁₉ molecule that is recognized in the context of *H-2^k*. These results highlight the significance of choosing the appropriate expression system and fusion partners for vaccine molecules.

As MSP1₁₉-mediated protection is reliant on high-titer antibodies present at the time of challenge (12, 13), T-cell studies were conducted to ascertain T helper responses and to determine whether there was a correlation between the T-cell determinants recognized and the subsequent antibody-mediated protection. The proliferative T-cell responses differed in the strains of mice, with T cells from each strain exhibiting a

different pattern of response to yMSP1₁₉ peptides (Fig. 3). B10.D2 and BALB/c mice express the same alleles at the *H-2* loci, yet it is interesting that T cells from B10.D2 mice respond only to peptide 24, while T cells from BALB/c mice respond to peptides 18 and 24. It is possible that regions outside the major histocompatibility complex (MHC) are also influencing the immune response to the peptides. The role of MHC- and non-MHC-associated genes in determining the immune response to malaria antigens was the subject of a recent review (25). The peptides recognized by B10.S mice after protein immunization are cryptic epitopes within yMSP1₁₉, as immunization with the recombinant protein did not produce a response to peptide 22 or 23. When the T-cell responses were examined in parallel with protection studies, there appeared to be no association between T-cell recognition of a particular peptide after peptide immunization and protection after parasite challenge. This is in keeping with the previous observation of Tian et al. (30) that vaccination of mice with defined T-cell epitopes of MSP1₁₉ would not protect them from parasite challenge. In the analysis of this experiment, we cannot exclude the possibility that one or more *H-2*-restricted epitopes were not represented by the series of overlapping peptides.

A successful vaccine against malaria will be able to immunize across a wide range of MHC class II haplotypes and elicit high levels of protection in its recipients. MHC-restricted responses are a concern, particularly in subunit vaccines where the necessary T-cell epitope(s) may not be incorporated. Vaccines containing MSP1₁₉ will also be restricted by the ability of T cells to recognize and respond to the T-cell epitopes within the protein. Studies performed with *P. falciparum* MSP1₁₉ demonstrated that T-cell responses to the protein were found in only 26% of naturally infected individuals tested and suggested that the complex structure of the native protein may be inhibiting antigen processing or presentation (9). This illustrates the need to develop strategies to overcome nonresponsiveness. The present study linked DT, a source of T helper epitopes, to yMSP1₁₉ in an attempt to overcome the MHC-linked nonresponsiveness observed in *H-2^s* mice. The carrier, DT, primed T helper cells for a secondary response to yMSP1₁₉. This phenomenon of carrier priming has previously been reported for different conjugates (23, 29). This antigenic modification of yMSP1₁₉ resulted in an immunogenic conjugate which enabled *H-2^s* mice to respond without eliminating the responses observed in another mouse haplotype (*H-2^b*).

Although both mouse strains immunized with the conjugates controlled parasitemia, the mice exhibited different levels of protection, with the B10.S mice having higher levels of parasitemia (Fig. 7). The higher peak parasitemia in the B10.S mice compared to the C57BL/10 mice is most likely a reflection of the lower antibody titers in this mouse strain.

The C57BL/10 mice that received yMSP1₁₉ alone did not develop patent parasitemia, yet the mice immunized with the

FIG. 7. Immunogenicity of yMSP1₁₉-DT conjugates. yMSP1₁₉-specific antibody titers (left axis, closed symbols) and the course of parasitemia (right axis, open symbols) in C57BL/10 (left) and B10.S (right) mice immunized with yMSP1₁₉, yMSP1₁₉-DT (MCS), yMSP1₁₉-DT (SPDP), DT, or PBS. Mice were challenged i.v. with 10⁴ *P. yoelii* YM parasitized red blood cell on day 127. Antibody titers and parasitemias were determined for the duration of the experiment, which concluded on day 155. Termination of the parasitemia and antibody titer curves on the graphs before day 155 indicates that the mouse succumbed to malaria infection and is represented by daggers.

FIG. 8. Ability of the immunizing antigens in the conjugation experiments to generate antibodies capable of affecting parasite growth and to bind to known protective epitopes. (A) Prepatent periods for the experimental groups. The median prepatent period of each group is indicated by a cross. Blood smears were examined until 30 days after parasite challenge, and mice that had not developed parasitemia by this time were allocated a prepatent period of 30 days. (B) Ability of sera from the experimental groups to inhibit the binding of MAb 302 to yMSP1₁₉. The median percent inhibition of each group is indicated by a cross. Values for experimental groups that were significantly different ($P < 0.05$) from the values for both control groups (mice immunized with DT or PBS and B10.S mice immunized with yMSP1₁₉) are indicated by asterisks.

conjugates developed low-level, controlled parasitemia (Fig. 7). The mice immunized with the conjugates also had a lower mean yMSP1₁₉-specific antibody titer than the mice immunized with yMSP1₁₉ alone. The course of parasitemia was in

part influenced by the mouse strain and the amount of antibody present at the time of challenge, which is related to the total amount of yMSP1₁₉ received in the immunizations. The C57BL/10 mice immunized with yMSP1₁₉ received 20 μ g of

FIG. 9. Relationship between the ability of the antibodies in the sera from the different experimental groups to inhibit the binding of MAb 302 to yMSP1₁₉ and affect the length of the prepatent period in C57BL/10 and B10.S mice. Mice were immunized with yMSP1₁₉-DT (MCS) (●), yMSP1₁₉-DT (SPDP) (▽), and yMSP1₁₉ (■) and with DT (◇) and PBS (▲) as controls.

the recombinant protein, while the mice immunized with the conjugate received 20 μ g of total protein, which included both yMSP1₁₉ and DT. A great amount of the antibody produced by the mice receiving the conjugate was specific for DT (data not shown) and may have diminished the MSP1₁₉-specific response. Increasing the total amount of conjugate given to the mice may result in a greater antibody response to yMSP1₁₉.

The rationale for the addition of T-cell epitopes to subunit vaccines is to increase the percentage of the population that can respond. After vaccination with an antigen coupled to an unrelated T-cell epitope, the level of boosting that would result from natural infection is unclear; however, in individuals who could not respond to the antigen alone, boosting would not be

predicted. The most likely outcome of vaccination with an MSP1₁₉ conjugate would be the production of a high titer of antibody that would suppress the parasitemia to a low level. This reduction in parasite burden would allow the recipient of the vaccine to survive the infection and give the recipient time to develop additional parasite-specific immune responses independent of the vaccine (14). The results presented here expand upon a previous study that expressed a GST fusion protein containing MSP1₁₉ with a defined T-cell epitope inserted between GST and MSP1₁₉ (1).

While the phenomenon of epitope-specific suppression (11, 27) should be addressed when using proteins, such as DT and tetanus toxoid, as a carrier for haptens, the carrier priming

FIG. 10. Effects of preexisting DT-specific antibodies on a subsequent immune response to yMSP1₁₉ in C57BL/10 and B10.S mice. The yMSP1₁₉-specific antibody titers (circles) and DT-specific antibody titers (squares) in mice preimmunized with DT (closed symbols) or PBS (open symbols) in CFA. The mice were given booster doses of yMSP1₁₉ in IFA 21 days later. Day 21 of the immunization schedule is shown as day 0 on these graphs. Each point represents the mean $\pm$ 95% confidence intervals (error bars) for five mice.

experiment in this study showed that the presence of an existing immune response to DT enhanced rather than suppressed a subsequent immune response to yMSP1₁₉ when yMSP1₁₉ was delivered as a DT conjugate (Fig. 10). It is probable that the initial immunization with the carrier, DT, resulted in the expansion of a population of T helper cells that upon subsequent immunization with the yMSP1₁₉-DT conjugate, facilitated a more rapid yMSP1₁₉-specific antibody response. These observations suggest that the presence of a DT-specific immune response in humans vaccinated by the normal vaccination regimen may in fact assist the development of an immune response to yMSP1₁₉ when delivered in the form of a DT conjugate. Further studies will be conducted to ascertain whether an immunization regimen consisting of a carrier preimmunization followed by multiple conjugate immunizations can induce a protective immune response.

When considering the use of vaccines in humans, it is necessary to address safety issues. Human studies have been undertaken to examine the side effect profile of immunizations containing carrier proteins, such as DT and tetanus toxoid (16, 24). Both studies observed that hypersensitivity reactions tended to occur after the third immunization with the conjugate and proposed that changes to formulation and immunization schedule (specifically the time between doses) may improve the safety profile. The immunization strategy outlined in the present study was a proof-of-principle study in mice, addressing issues, such as immunogenicity and epitopic suppression versus carrier priming. If further studies were to demonstrate that these conjugates were immunogenic in humans, a possible strategy to overcome hypersensitivity reactions induced by multiple doses would be to use MSP1₁₉-DT in place of DT in the normal vaccine schedule. This could simultaneously limit the number of additional immunizations required and exploit any preexisting immune response to the carrier, further enhancing the conjugate's immunogenicity.

In conclusion, it was possible to overcome the genetic restriction seen in B10.S mice when immunizing with yMSP1₁₉. The addition of helper T epitopes present in DT to yMSP1₁₉ resulted in an immunogen that was able to induce antibodies and protective immunity in previously totally nonresponsive, *P. yoelii*-susceptible mice. DT may be a preferred conjugate partner for MSP1₁₉, as prior exposure to DT subsequently enhances the immune response to the conjugate. These results may have an important application when considering ways to enhance the immunogenicity of *P. falciparum* yMSP1₁₉ vaccines for use in the human population.

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