Merozoite Surface Protein 1-Specific Immune Response Is Protective against Exoerythrocytic Forms of *Plasmodium yoelii*

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One of the difficulties in developing an effective malaria vaccine is the antigenic change of the parasite during the life cycle. It is desirable that vaccine-induced protective immunity be effective at different stages of parasite development. Merozoite surface protein 1 (MSP1) is a candidate vaccine antigen against blood-stage malaria, but it is also expressed in the exoerythrocytic forms. It was not known, however, whether the anti-MSP1 immune response is effective against the liver-stage malaria parasite. We generated a recombinant protein of MSP1 fused to heat-shock cognate protein 70 (hsc70) and studied its vaccination effect. When C57BL/6 mice were immunized with the fusion protein prior to challenge infection with *Plasmodium yoelii* sporozoites, the onset of parasitemia was delayed or no parasitemia was observed. To determine whether this was due to the protective immunity against liver-stage parasites, *P. yoelii*-specific rRNA in the infected liver was quantitated by real-time reverse transcription-PCR analysis. The level of parasite-specific rRNA was reduced in mice immunized with the fusion protein of MSP1 and hsc70 but not with hsc70 alone, indicating that MSP1-specific immune immunity can be protective against the exoerythrocytic form of the parasite. Furthermore, the adoptive transfer experiments of immune lymphocytes and serum into naive mice suggested that the protective immunity was dependent on cellular and not humoral immunity. Finally, the vaccine-induced protection was also observed in A/J, C3H, and BALB/c mice, suggesting that MSP1-specific protective immunity at the exoerythrocytic stage can be induced in animals over a wide range of genetic backgrounds.

Mice immunized with irradiated sporozoites become protected against exoerythrocytic (EE) forms of the malaria parasite, which develop in the liver from sporozoites (15). This protective immunity is mediated at least in part by CD8 T cells and gamma interferon (IFN-γ) (17). Several parasite antigens that are expressed in the sporozoite or EE forms and that can induce protective immunity have been identified as potential targets of protective immunity; these include circumsporozoite protein, sporozoite surface protein 2 (SSP2), and liver-stage-specific antigens 1 and 2 (LSA-1 and LSA-2) (14). Protective immunity targeted to these antigens can induce sterile immunity against sporozoite infection. However, the weakness of these vaccines is that they are ineffective once the blood infection is established. It is desirable for the protective immunity to be effective at both the liver and blood stages of the malaria life cycle. Some malaria antigens are expressed at both the EE and blood stages of parasite (1). One of them is merozoite surface protein 1 (MSP1), a prominent candidate antigen for a blood-stage malaria vaccine. Using monoclonal antibodies specific for MSP1, the precursors and proteolytic products of MSP1 were shown to be expressed in the EE stage (19, 20). MSP1 is synthesized as a precursor of 180 to 250 kDa and is bound to the merozoite plasma membrane via a C-terminal glycosyl-phosphatidylinositol moiety (9). Maturation of this precursor by a two-step proteolytic cleavage results in the 19-kDa fragment of MSP1, comprised of two epidermal growth factor-like domains that are attached on the surface of merozoites (3, 5).

Immunizations of mice with malaria antigens have been performed using a variety of adjuvants, including Freund's adjuvant (16, 22), Rabi adjuvant (6), recombinant BCG (12), and DNA vaccine (2, 11, 14). Heat shock protein 70 (hsp70) is a molecular chaperon which can induce both CD4- and CD8-mediated immune responses against its associated antigens (18). We and others showed previously that antigens fused to hsp70 or heat-shock cognate protein 70 (hsc70) can induce CD8 T-cell responses (10, 24). In this study, we generated a recombinant protein of MSP1 fused to murine hsc70 and studied whether it could induce protective immunity. The results showed that MSP1-specific immune responses could be protective against EE forms of malaria parasites.

**MATERIALS AND METHODS**

**Animals and parasites.** Female C57BL/6, A/J, C3H, and BALB/c mice were purchased from SLC (Hamamatsu, Japan) and were maintained in the Laboratory Animal Center for Biomedical Research at Nagasaki University School of
Medicine. *P. yoelii* 17XL and *Anopheles stephensi* were provided by M. Torii (Ehime University, Japan). *P. yoelii* 17XL was maintained by alternating passage between *A. stephensi* and DBA/2 mice. The animal experiments reported herein were conducted according to the guidelines of the Laboratory Animal Center for Biomedical Research, Nagasaki University. RNA from infected hepatocytes was isolated using Isogen (Nippon Gene, Tokyo, Japan).

**Recombinant fusion protein.** The MSP1 C-terminal 15-kDa fragment was amplified by PCR from *P. yoelii* msp1 cDNA (a gift of S. Matsumoto, Department of Bacteriology, Dental School, Nagasaki University) by using a pair of primers, 5'-AAAGATCCCATACAGTCTACATGCT and 5'-ACCCTGCACTCCTCAAAAGCTGAAG, to generate BamHI and SalI sites (underlined), respectively. cDNA of 10-mm punching mouse hsc70 was isolated (24), and SalI and HincII sites were introduced by PCR using 5'-CAGGTGCACATGTCATAAGGGACCTGC and 5'-CACAGAATTCTATACCCCTCCA, respectively. Each product was digested with BamHI/SalI or SalI/HincII and subcloned into the expression vector pQE31 (Qiagen, Hilden, Germany). The expression of MSP1-hsc70 was induced in the expression vector pQE31 (Qiagen, Hilden, Germany). The expression of MSP1 in naive recipient C57BL/6 mice. The parasite burden of the individual livers was determined by real-time RT-PCR. The primer pair used to amplify cDNA of 18S rRNA was 5'-ACATGGCTATGCAGGCTTCTCC (200 nM) and 5'-CCCTTCATTAGTGATGCAATTC (50 nM). Mouse G3PDH was also expanded in parallel using the pair of primers 5'-CATCTGAGGCCACTTGAAG (200 nM) and 5'-TGCTGTGGAACGCG (200 nM). The threshold cycle of each PCR was converted to a DNA equivalent by reading against standard curves generated by amplifying 10-fold dilutions of plasmid containing the relevant target molecules. The liver parasite burden was determined as the ratio of the DNA equivalent measured for the *P. yoelii* 18S rRNA over the DNA equivalent for mouse G3PDH.

**Lymphocyte preparation and adoptive transfer.** Hepatic mononuclear cells were prepared as previously described (7, 25). Briefly, the liver was removed from a mouse sacrificed by total bleeding via the axillary artery and vein. The hepatic mononuclear cells were prepared by pressing the liver through a stainless steel mesh and suspending them in RPMI medium containing 2% fetal calf serum. After washing once, the pellet was resuspended in a 30 to 35% Percoll solution and centrifuged for 15 min at 750 × g at room temperature. After washing once, the pellet was resuspended in red blood cell lysis solution and washed twice with medium.

Spleen cells and hepatic lymphocytes collected from each group of donor mice 2 weeks after the final immunization (five mice/group) were separately pooled and transferred i.v. via the tail vein into naive recipient C57BL/6 mice. Spleen cells from mice not immunized or immunized with MSP1-hsc70 (10⁴) were adoptively transferred via the tail vein into naïve syngeneic recipients (five mice/group). Hepatic lymphocytes from immunized mice (10⁴) or nonimmunized mice (2 × 10⁶) were also transferred into naïve recipients. In another group each mouse received an i.v. injection of 0.4 ml of antisera obtained from mice immunized with MSP1-hsc70. Two days after the adoptive transfer, the recipients were challenged with 1,000 infectious *P. yoelii* sporozoites. Forty-eight hours after the challenge, mice were sacrificed to collect serum and the liver. The parasite burden of the individual livers was determined by real-time RT-PCR. The titer of serum Abs specific for MSP1 was determined by ELISA.

**Statistics.** Significance levels determined by Mann-Whitney’s U test for unpaired observations. The result was considered significant when the P value was less than 0.05.

**RESULTS**

Expression of *P. yoelii* MSP1 at the liver stage. MSP1 is a leading candidate antigen for a malaria vaccine at the blood stage, but its expression is also reported in the EE forms of malaria infection (19, 20). To confirm the expression of MSP1 during the liver stage, we performed RT-PCR of liver RNA 0 to 4 days after sporozoite infection (Fig. 1). Expression of MSP1 mRNA was detected 2 days after sporozoite infection. The levels of MSP1 mRNA in the infected liver decreased 3 and 4 days after infection, corresponding to the release of merozoites from the hepatocytes. MSP1 mRNA expressed 2 days after infection should derive from the infected hepatocytes, although those expressed 3 and 4 days after infection may contain parasite RNA from the infected hepatocytes and
red blood cells. The result confirmed that MSP1 is expressed by the EE forms of *P. yoelii*.

**Induction of MSP1-specific immune responses by MSP1-hsc70 fusion protein.** Hsp70 can induce both CD4- and CD8-mediated immune responses against its associated antigens without any additional adjuvant (18). Therefore, we generated a recombinant protein in which the C-terminal 15-kDa fragment of MSP1 was fused to mouse hsc70 (Fig. 2A). The recombinant MSP1-hsc70 fusion protein and control mouse hsc70 were expressed in *E. coli* and purified using Ni$^{2+}$ affinity chromatography (Fig. 2B).

C57BL/6 mice were immunized i.v. with MSP1-hsc70 or hsc70 alone without any additional adjuvant at intervals of 2 weeks. This route of immunization was chosen because it can induce specific cytotoxic T-cell responses most efficiently (24).

Two weeks after the third immunization, production of MSP1-specific Abs and cytokines was assessed. Antiserum was obtained from each immunized mouse, and the relative concentrations of anti-MSP1 IgG, IgM, IgG1, IgG2a, IgG2b, and IgG3 were determined at a dilution of 1:500 by ELISA using a plate coated with GST-MSP1. Mice immunized with MSP1-hsc70 showed high levels of anti-MSP1 Abs of IgG1, IgG2a, and IgG2b subclasses (Fig. 3A). We also examined whether T cells from the immunized mice could produce IFN-γ in response to MSP1, since IFN-γ is a critical cytokine for the protective immune responses against liver-stage malaria parasites (17). Two weeks after the final immunization, spleen cells were prepared from mice immunized with MSP1-hsc70 or hsc70 and cultured for 24 h in the presence and absence of GST-MSP1, and the numbers of IFN-γ-producing cells were determined by ELISPOT assay (Fig. 3B). The number of splenocytes producing IFN-γ in response to MSP1 increased significantly in mice immunized with MSP1-hsc70 compared with those immunized with hsc70 alone. No significant production of interleukin-4 was detected in these cells (data not shown). These results indicated that MSP1-specific humoral immune responses and IFN-γ production were induced in mice immunized with MSP1-hsc70 without additional adjuvant.

**Lessening of parasitemia in mice immunized with MSP1-hsc70.** We next evaluated whether protective immunity against sporozoite infection was induced by immunization with MSP1-hsc70. Immunized and nonimmunized C57BL/6 mice were challenged with 50 viable sporozoites, and parasitemia of the mice was monitored daily after sporozoite challenge. When mice were not immunized, parasitemia became detectable in blood smears 4 to 5 days after sporozoite infection, increased in a logarithmic manner, and led to the death of essentially all mice within 20 days (Fig. 4A). One mouse did not develop parasitemia in this particular experiment, for unknown reasons. When mice were immunized with hsc70 alone, most mice developed parasitemia with kinetics similar to nonimmunized mice, although a slight delay in the development of parasitemia was observed in some mice and one mouse did not develop significant parasitemia (Fig. 4B). When mice were immunized with MSP1-hsc70, the appearance of parasitemia was quite different. No parasitemia was observed in four out of five immunized mice for at least 20 days after infection. When parasitemia appeared, it increased to ~40% and declined in most immunized mice, suggesting that protective immunity against the blood stage was also elicited (Fig. 4C). In repeated experiments, we consistently observed either a delay in the onset of parasitemia by 2 to 4 days or no apparent parasitemia after sporozoite infection in mice immunized with MSP1-hsc70.
Induction of protective immunity against the EE form of parasites by immunization with MSP1-hsc70. The delay in the onset of parasitemia after sporozoite challenge generally corresponds to a reduction in the number of liver forms (13). Thus, we considered it likely that the protective immunity was acquired at the liver stage in mice immunized with MSP1-hsc70. To investigate this possibility, levels of EE forms in the liver were determined by RT-PCR analysis of malaria-specific 18S rRNA (4). In naive C57BL/6 mice that were challenged with 1,000 sporozoites, the level of P. yoelii rRNA in the liver dramatically increased 2 days after sporozoite infection, declined on day 3, and increased again on day 4 (Fig. 3A). The reduction and the subsequent increase of rRNA levels may reflect the release of merozoites into the blood from hepatocytes and the growth of blood forms within the liver circulation. We therefore measured parasite burden in the liver 2 days after the challenge infection. C57BL/6 mice immunized four times i.v., or not immunized with MSP1-hsc70 or hsc70, received 1,000 sporozoites 2 weeks after the last immunization, and total liver RNA was extracted from individual mice 2 days after the infection. Each group contained five mice. One set of representative data from several experiments with similar results is shown.

![Graph showing the course of parasitemia in C57BL/6 mice not immunized (A) or immunized with hsc70 (B) or MSP1-hsc70 (C) and challenged with P. yoelii 17XL sporozoites. Mice were immunized seven times i.v. with hsc70 or MSP1-hsc70. Two weeks after the last immunization, groups of five mice were challenged with 50 P. yoelii 17XL sporozoites i.v. The parasitemia of each mouse was monitored daily after day 4 of the infection. Each group contained five mice. One set of representative data from several experiments with similar results is shown.](http://iai.asm.org/)

![Graph showing MSP1-specific Ab and IFN-γ responses induced by immunization with MSP1-hsc70. (A) MSP1-specific Ab isotypes in the serum of C57BL/6 mice not immunized (white bar) and immunized three times intravenously with hsc70 (gray bar) or MSP1-hsc70 (black bar). The levels of anti-MSP1 Ab subclasses were determined by ELISA at a serum dilution of 1:500 using anti-mouse subclass-specific Abs. The data shown are the mean ± standard deviation of five mice in each group and represent three independent experiments with similar results. (B) The number of IFN-γ-producing cells in the spleens of C57BL/6 mice not immunized (white bar) and immunized three times i.v. with hsc70 (gray bar) or MSP1-hsc70 (black bar) was determined by ELISPOT assay. Spleen cells were recovered from these mice 2 weeks after the final immunization and cultured for 24 h in the presence and absence of GST-MSP1 for ELISPOT assay. Values represent the number of IFN-γ-producing cells per 10⁶ splenocytes stimulated with GST-MSP1 minus the number of those stimulated with PBS only (37, 66, and 41 spleen cells in mice not immunized, immunized with hsc70, and with MSP1-hsc70, respectively). The results are expressed as the mean ± standard deviation of triplicate cultures and represent two independent experiments with similar results.](http://iai.asm.org/)
Protection against EE forms of parasites in different strains of mice immunized with MSP1-hsc70. It has been reported that protective immunity against EE forms of malaria parasites by immunization with irradiated \textit{P. yoelii} sporozoites is genetically controlled (26). MSP1 vaccine-induced protection against blood-stage parasites is also genetically controlled (22). Therefore, we evaluated the effect of immunization with MSP1-hsc70 by using three different strains of mice, A/J (H-2^d), C3H (H-2^k), and BALB/c (H-2^d). Two weeks after the third immunization, mice were challenged with \textit{P. yoelii} sporozoites and parasitemia was monitored daily. A/J mice were relatively resistant to \textit{P. yoelii} infection. The levels of parasitemia did not reach 50% and declined by 2 weeks after infection. When A/J mice were immunized with MSP1-hsc70, the onset of parasitemia was delayed for ~2 days and the parasitemia did not reach more than 2% (Fig. 7A). In contrast, infection was lethal in both C3H and BALB/c mice. When
FIG. 6. Adoptive transfer of lymphocytes and antisera from C57BL/6 mice immunized with MSP1-hsc70 into naive recipient mice. (A) C57BL/6 mice not immunized or immunized with MSP1-hsc70 seven times were used as donors. Two weeks after the last immunization, spleen cells and hepatic lymphocytes were collected from the donor mice (five mice/group) and transferred into naive recipient mice (five mice/group). Two days after the transfer, mice were challenged with 1,000 sporozoites and liver parasite burden was assessed as described in Materials and Methods. The data shown are the mean ± standard deviation of five mice in each group and represent two experiments with similar results. (B) Levels of anti-MSP1 Ab in the sera of mice used in the experiment shown in panel A. Two days after the sporozoite challenge, serum was obtained from each mouse prior to sacrifice for the removal of its liver. The levels of anti-MSP1 Ab in the sera were assessed by ELISA after serial dilution. The data shown are the mean ± standard deviation for five mice in each group and represent two experiments with similar results.

These mice were immunized with MSP1-hsc70, the appearance of parasitemia was delayed by ~2 days, although all of them eventually died after high levels of parasitemia (Fig. 7B and C). The delay in the onset of parasitemia in the immunized mice suggested that the protective immunity is effective at the liver stage in these strains. To examine this possibility, the parasite burden in the liver was determined 2 days after sporozoite challenge by real-time PCR (Fig. 7D and E). In both C3H and BALB/c mice, liver parasite burden in immunized mice was reduced, suggesting that immunization of these strains of mice with MSP1-hsc70 induced protective immunity against the EE forms of P. yoelii.

**DISCUSSION**

MSP1 is expressed not only at the blood stage but also in EE forms of malaria parasites (19, 20). In this study we confirmed the expression of MSP1 in the liver by RT-PCR and investigated whether this antigen could be a target of protective immunity against liver-stage parasites by an immunization protocol that used MSP1-hsc70 fusion protein. Immunization of mice with MSP1-hsc70 induced humoral and cellular immune responses specific for MSP1. When these mice were challenged with P. yoelii sporozoites, some mice showed late onset of parasitemia and others showed no apparent parasitemia. We examined a possibility that anti-MSP1 immune responses inhibited the expansion of EE forms within the liver, since the delay in the onset of parasitemia after sporozoite challenge generally correlates to a reduction in the number of liver forms (reference 13 and our unpublished data). To estimate the number of parasites in the liver, we performed real-time RT-PCR analysis of P. yoelii-specific rRNA. The levels of parasite RNA were reduced in mice that were immunized with MSP1-hsc70 but not those immunized with hsc70 alone, indicating that the MSP1-specific immune response could be protective against EE forms of P. yoelii. Furthermore, the adoptive transfer experiment ruled out possible involvement of the preexisting anti-MSP1 Ab in the protective immunity against EE forms of parasites and indicated that this protective immunity within the liver was mediated by cellular and not by humoral immunity. Thus, MSP1-specific cellular immune responses can be protective against EE forms of P. yoelii. This conclusion does not, however, necessarily contradict the contribution of anti-MSP1 Ab to the inhibition of parasite development at the blood stage right after their emergence from the liver. Rather, it is more likely that both cellular immune responses against the liver stage and humoral immune responses against the blood stage contribute to the delayed onset of parasitemia and the subsequent recovery.

A vaccine that induces protective immunity against both the EE and blood stages would increase the chance of protection. The immune response against the parasite antigens could be boosted during the liver-stage infection and could eliminate parasites more efficiently during the blood stage even if the emergence of malaria parasites into the bloodstream could not be completely prevented. To achieve this boosting effect, lymphocyte types that promote protection during the blood-stage infection should be the same as those stimulated during the liver-stage infection. In this regard, CD4+ T cells have been shown to be involved in protective immunity at both the liver and blood stages (8, 23). Therefore, it might be possible to design a vaccine that induces protective immune responses effective at both the liver and blood stages of malaria infection. In the present study, we showed that mice immunized with MSP1-hsc70 became protected against challenge infection of P. yoelii sporozoites not only at the blood stage but also at the liver stage of infection. In addition, the adoptive transfer of protective immunity at the liver stage by lymphocytes of the immunized mice into naïve recipients indicated cellular mechanisms of the protection against EE forms of the parasites. However, the detailed mechanisms of the protection at the liver stage are not clear. Immunization of mice with MSP1-hsc70 induced specific T cells that produce IFN-γ. The main source of IFN-γ appears to be CD4+ T cells, since the treatment of spleen cells with anti-CD4 Ab and complement removed most of the IFN-γ-producing cells (data not shown). However, the depletion of CD4+ cells by inoculation of anti-
CD4 Ab in vivo in mice immunized with MSP1-hsc70 did not result in the loss of protection at the liver stage, while it eliminated the protection at the blood stage (data not shown). Thus, cell types that mediate the protective immunity against EE forms of *P. yoelii* appear distinct from those involved in blood-stage protection. We are currently investigating the cellular mechanisms of the protective immunity induced by MSP1-hsc70 against EE forms of *P. yoelii* infection.

Protective immune responses of mice vaccinated with MSP1 against blood-stage malaria parasites are genetically regulated (22). The protective ability is linked to major histocompatibility complex-linked loci and is related to levels of protective Ab and T-cell cytokine production. In agreement with these earlier findings, we observed differences in the protection of different strains of mice that were immunized with MSP1-hsc70 (Fig. 7 and data not shown). C57BL/6 and A/J mice were protected at blood stages, eventually resolving the infection, while both C3H and BALB/c mice died after high levels of parasitemia. These susceptible mice, however, showed a delay in the onset of parasitemia after immunization. In addition, the liver parasite burden of these strains of mice that were immunized with MSP1-hsc70 was reduced compared with that in nonimmunized mice. Thus, the protection against EE forms could be induced in animals over a wider range of genetic backgrounds, including those that are relatively low responders to the vaccine against blood-stage malaria. These differences in the protective immunity induced by immunization with MSP1-hsc70 against liver- and blood-stage malaria among different strains of mice were investigated further.
strains of mice also suggest that there might be differences in the mechanisms of protection against these two forms of infection, as we have discussed.

MSP1 is a leading candidate antigen for malaria vaccines and its efficacy has been evaluated in many studies. Most studies used parasitized blood for challenge infection, and those using sporozoites are limited. In one study, sporozoites were used to challenge BALB/c mice that were immunized with MSP1 in complete Freund’s adjuvant (16). In another, sporozoites were used to challenge BALB/c and C57BL/6 mice that were immunized with MSP1 DNA vaccine (2). In both studies, some of the immunized mice did not develop any patent blood-stage parasitemia after sporozoite infection, but it was not clear whether this was due to the protection against EE forms of the malaria parasites. In this study, we showed that MSP1-specific immune responses could be protective against EE forms. It would be desirable to induce maximal protective immunity at both the EE and blood stages when designing an MSP1 vaccine delivery system.

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