INFECTION AND IMMUNITY, Nov. 2002, p. 6075–6082 Vol. 70, No. 11
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.11.6075–6082.2002

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

Yuko Kawabata,[1] Heiichiro Udono,[1] Kiri Honma,[1] Masakatsu Ueda,[1] Hiroshi Mukae,[2] Jun-ichi Kadota,[2] Shigeru Kohno,[2,3] and Katsuyuki Yui[1]*

Division of Immunology, Department of Molecular Medicine, Nagasaki University School of Medicine,[1] and Division of Molecular and Clinical Microbiology, Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Medical Sciences,[3] 1-12-4, Sakamoto, Nagasaki 852-8523, and Second Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501,[2] Japan

Received 22 April 2002/Returned for modification 11 June 2002/Accepted 6 August 2002

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 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 glycosylphosphatidylinositol 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). Immunization of mice with a 15-kDa portion of this C terminus of *Plasmodium yoelii* MSP1 induces protective immunity against a lethal challenge of parasitized erythrocytes (6, 12). It has not been shown, however, whether MSP1-specific protective immune responses are effective against EE forms of the malaria parasites.

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.

* Corresponding author. Mailing address: Division of Immunology, Department of Molecular Medicine, Nagasaki University School of Medicine, 1-12-4, Sakamoto, Nagasaki 852-8523, Japan. Phone: 81-95-849-7070. Fax: 81-95-849-7073. E-mail: katsu@net.nagasaki-u.ac.jp.

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 School of Medicine.

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′-AAGGATCCACACATAGCCTCAATAGCT and 5′-ACCGTCGACTCCCATAAAGCTGGAAG, to generate *Bam*HI and *Sal*I sites (underlined), respectively. cDNA encoding mouse hsc70 was isolated (24), and *Sal*I and *Hin*dIII sites were introduced by PCR using 5′-CAGGTCGACATGTCTAAGGGACCTGC and 5′-CACAAGCTTAATCCACCTCTTCAA, respectively. Each product was digested with *Bam*HI/*Sal*I or *Sal*I/*Hin*dIII and subcloned into the expression vector pQE31 (Qiagen, Hilden, Germany). The expression of MSP1-hsc70 and hsc70 was induced in *Escherichia coli* M15 with 1 mM isopropyl-β-D-thiogalactopyranoside and purified using Ni^{2+} affinity chromatography under denaturing conditions as described previously (24). Briefly, bacteria were lysed in phosphate buffer (0.1 M, pH 8.0) containing 8 M urea at room temperature. After centrifugation, the supernatant was applied to a Ni-nitrilotriacetic acid agarose column (Qiagen) and washed extensively with phosphate buffer (0.1 M, pH 6.3) containing 8 M urea, followed by urea-free Tris buffer (pH 7.5) and phosphate-buffered saline (PBS; pH 7.4). The recombinant protein was eluted with PBS containing 200 mM imidazole and dialyzed extensively with PBS. MSP1 cDNA was also subcloned into the plasmid pGEX2T to obtain a recombinant fusion protein of *Schistosoma japonicum* glutathione *S*-transferase and MSP1 (GST-MSP1). *E. coli* XL1 Blue was transformed, and GST-MSP1 was isolated from bacterial lysates by affinity chromatography according to the manufacturer's instructions (Pharmacia Biotech, Inc.) (12). The endotoxin content of the purified recombinant protein determined by Limulus test was less than 0.5 ng/mg of protein.

Immunization and infection. Mice were immunized intravenously (i.v.) via the tail vein with 10 μg of MSP1-hsc70 or hsc70 recombinant proteins without additional adjuvant, three to seven times at 2-week intervals. Two weeks after the last immunizations, mice were challenged with 50 or 1,000 infectious sporozoites which were obtained from salivary glands of *P. yoelii* 17XL-infected mosquitoes. Sporozoites were injected in each mouse in 0.2 ml of M199 for parasitemia and reverse transcription-PCR (RT-PCR) analysis of the infected liver RNA. The course of parasitemia was monitored by microscopic examination of Giemsa-stained tail-blood smears.

Measurement of anti-MSP1 Ab titer. Serum was collected from individual mice and stored at −20°C until use. The titer of antibody (Ab) specific for MSP1 was determined by enzyme-linked immunosorbent assay (ELISA). Each well of a microtiter plate (Dynatech, Hindenburgstrasse, Germany) was coated with GST-MSP1 (2 μg/ml) in 50 μl of binding buffer (0.1 M Na_2HPO_4, pH 9.0) by incubation at room temperature for 2 h. The wells were blocked with 200 μl of blocking buffer (10% fetal calf serum, 0.02% NaN_3 in PBS) for 1 h and washed with PBS containing 0.05% Tween 20. Serum was diluted (1:500) with blocking buffer containing 0.05% Tween 20 and placed in each well of a microtiter plate (100 μl/well) for 2 to 4 h. After washing with PBS containing 0.05% Tween 20 (PBS-Tween), each well was filled with 100 μl of blocking buffer containing alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG; Sigma) and incubated at room temperature for 1 h. For isotype determination of anti-MSP1 Abs, biotinylated anti-mouse IgM, IgG1, IgG2a, IgG2b (Zymed, San Francisco, Calif.), and IgG3 (Pharmingen) Abs were used in conjunction with alkaline phosphatase-streptavidin. After washing with PBS-Tween, the plates were developed with a solution of alkaline phosphatase substrate (Sigma). The optical density at 405 nm of each well was measured using a plate reader.

IFN-γ-specific ELISPOT assay. Nitrocellulose-lined 96-well microtiter plates (MAHAS45; Millipore, Bedford, Mass.) were coated with 5 μg of anti-mouse IFN-γ Ab (clone R4-6A2; Pharmingen)/ml at room temperature for 2 h (21). After washing with PBS containing 0.25% Tween 20, plates were overlaid with PBS containing 5% bovine serum albumin at 37°C for 30 min. Spleen cells were prepared from mice 2 weeks after the final immunization and placed in each well of a treated microtiter plate (5×10^5 to 10×10^5 cells/100 μl) in the presence and absence of an antigen and incubated at 37°C for 24 h in a humidified atmosphere of 5% CO_2. After washing thoroughly, plates were incubated with biotinylated anti-IFN-γ (clone XMG-1.2; PharMingen), washed, and incubated with streptavidin-conjugated alkaline phosphatase and then with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium. Spots were counted using a stereomicroscope with a magnification of ×20.

Detection of *P. yoelii* in the liver. Total liver RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan). cDNA was generated from 2 μg of RNA using random hexamers and Moloney murine leukemia virus reverse transcriptase (Sawady Technology, Tokyo, Japan). MSP1 cDNA was amplified using a pair of primers, 5′-AAGGATCCACACATAGCCTCAATAGCT and 5′-ACCGTCGACTCCCATAAAGCTGGAAG. The amplified products were confirmed by sequencing. Treatment of the RNA with DNase prior to RT-PCR analysis did not have any effect on the result. Also, no DNA product was observed in control PCR using samples generated without reverse transcriptase, confirming that the PCR product was not from contaminating genomic DNA. Parasite-specific 18S rRNA was amplified by PCR from 1 μl of cDNA mixture using specific primers as described previously (4). Mouse glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was amplified using the pair of primers 5′-ACCACAGTCCATGCCATCAC and 5′-TCCACCACCCTGTTGCTGTA and used as a control to evaluate the equal loading of the cDNA. PCR products were resolved on 2% agarose gels and visualized with ethidium bromide. Parasite-specific 18S rRNA was also measured by an automated real-time RT-PCR system using the ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, Calif.). Briefly, RNA isolated from each infected mouse liver was converted to cDNA and amplified by PCR using specific primers targeted to the 18S rRNA sequence of *P. yoelii* or mouse G3PDH in the PCR buffer containing the double-stranded DNA-specific fluorescent dye SYBR Green (Applied Biosystems). The primer pair used to amplify cDNA of 18S rRNA was 5′-ACATGGCTATGACGGGTAACG (100 nM) and 5′-CCTTCCTTAGATGTGGTAGCTATTTCTC (50 nM). Mouse G3PDH was also amplified in parallel using the pair of primers 5′-CATCTGAGGGCCCACTGAAG (200 nM) and 5′-TGCTGTTGAAGTCGCAGGAG (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, cells were 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 five naive recipient C57BL/6 mice. Spleen cells from mice not immunized or immunized with MSP1-hsc70 (10^8) were adoptively transferred via the tail vein into naive syngeneic recipients (five mice/group). Hepatic lymphocytes from immunized mice (10^7) or nonimmunized mice (2×10^6) were also transferred into naive recipients. In another group each mouse received an i.v. injection of 0.4 ml of antiserum 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 were 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

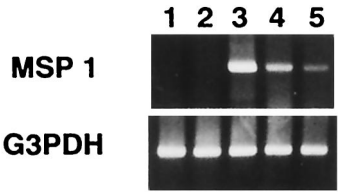

FIG. 1. Expression of MSP1 during the liver stage of *P. yoelii* 17XL infection. RNA was extracted from the liver of C57BL/6 mice without infection (lane 1) or 1 day (lane 2), 2 days (lane 3), 3 days (lane 4), or 4 days (lane 5) after i.v. inoculation with 1,000 viable sporozoites. RT-PCR analysis of *P. yoelii* MSP1 and control G3PDH mice was performed using specific primers. The data represent three separate experiments with similar results.

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.

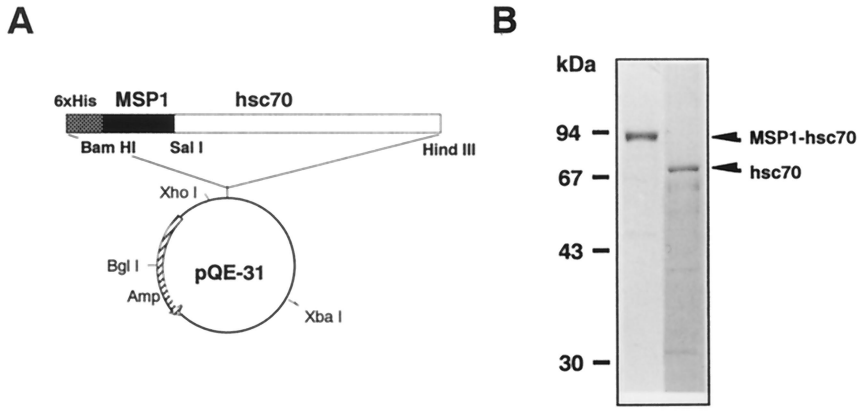

FIG. 2. Construction and purification of the MSP1-hsc70 fusion protein. (A) Plasmid map of the expression vector containing MSP1-hsc70. A DNA fragment of the C-terminal 15-kDa MSP1 antigen was fused to the N terminus of murine hsc70. (B) Recombinant MSP1-hsc70 fusion protein (85 kDa) and hsc70 were purified and resolved with sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis. The gel was stained with Coomassie brilliant blue.

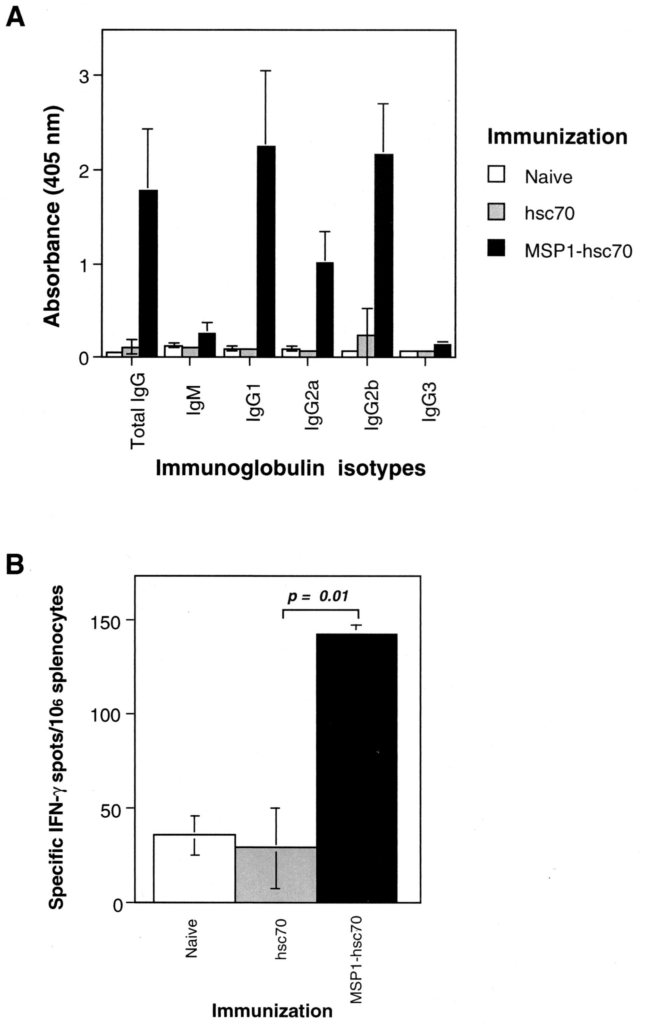

FIG. 3. MSP1-specific Ab and IFN-γ responses induced by immunization with MSP1-hsc70. (A) MSP1-specific Ab isotypes in the serum of C57BL/6 mice not immunized (white bar) and immunized three times intravenously with hsc70 (gray bar) or MSP1-hsc70 (black bar). The levels of anti-MSP1 Ab subclasses were 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 10^6 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.

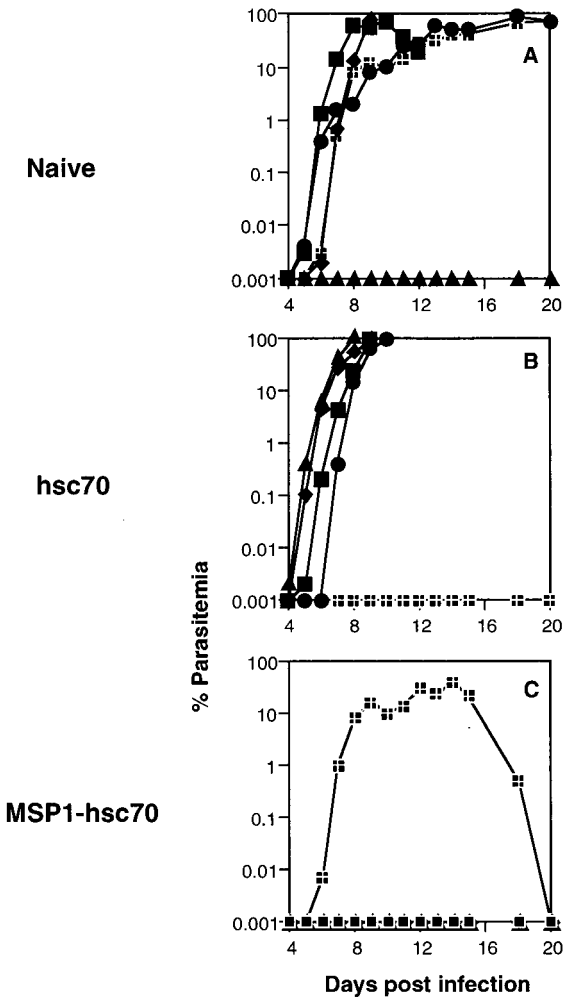

FIG. 4. The course of parasitemia in C57BL/6 mice not immunized (A) or immunized with hsc70 (B) or MSP1-hsc70 (C) and challenged with *P. yoelii* 17XL sporozoites. Mice were immunized seven times i.v. with hsc70 or MSP1-hsc70. Two weeks after the last immunization, groups of five mice were challenged 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.

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 of *P. yoelii* 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. 5A). The reduction and the subsequent increase of rRNA levels on days 3 and 4 of sporozoite infection 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

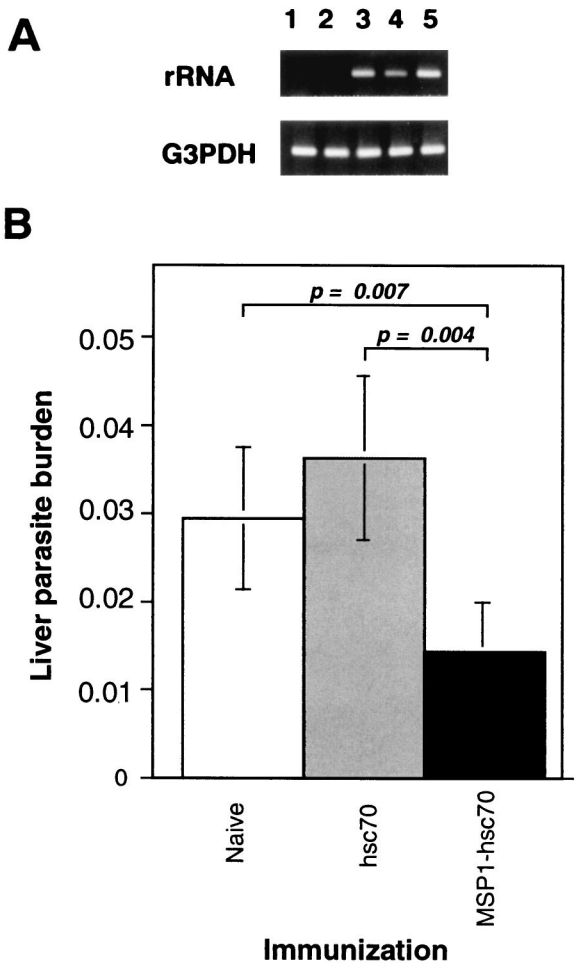

FIG. 5. Inhibition of *P. yoelii* development during the liver stage in C57BL/6 mice immunized with MSP1-hsc70. (A) The levels of *P. yoelii* rRNA in the liver were examined by RT-PCR on day 0 (not infected; lane 1), day 1 (lane 2), day 2 (lane 3), day 3 (lane 4), and day 4 (lane 5) after infection with 1,000 viable sporozoites. The data represent four experiments with similar results. (B) C57BL/6 mice were not immunized (white bar) or immunized four times i.v. with hsc70 alone (gray bar) or MSP1-hsc70 (black bar). Two weeks after the last immunization mice were challenged by i.v. injection of 1,000 sporozoites. Two days after the infection, RNA was extracted from each liver. The liver parasite burden was determined as the ratio of DNA equivalent measures for the *P. yoelii* 18S rRNA over the DNA equivalent for mouse G3PDH. The analysis included five SYBR Green measures from livers recovered from six mice per group. We performed three independent experiments with similar results, and the data represent pools of two experiments.

challenge. Their liver parasite burden (mean molecules of parasite rRNA/G3PDH) was assessed by automated real-time RT-PCR analysis (Fig. 5B). A significant reduction in the liver parasite burden was observed in mice immunized with MSP1-hsc70, compared with that in naive as well as hsc70-immunized mice, indicating that the immune responses induced by immunization with MSP1-hsc70 fusion protein were protective against the EE form of *P. yoelii* infection.

Adoptive transfer of the protective immunity into naive mice. Immunization of mice with MSP1-hsc70 induces both humoral and cellular immune responses. Therefore, we examined which branch of the immune response plays a major role in the protective immunity against EE forms of malaria parasites by performing adoptive transfer experiments of immune lymphocytes and antiserum. C57BL/6 mice were immunized seven times with MSP1-hsc70. Two weeks after the final immunization, spleen and hepatic lymphocytes were collected and were separately transferred into five naive syngeneic mice. Antiserum was also obtained from these mice for adoptive transfer (0.4 ml/mouse). In control groups, cells were collected from five nonimmunized mice and were transferred into naive mice in a similar manner. The number of cells transferred to each mouse was equivalent to the number of cells recovered from one spleen or liver of the donor mouse: 10^8 spleen cells and 2×10^6 hepatic lymphocytes of the naive mice, and 10^8 spleen cells and 10^7 hepatic lymphocytes of the immunized mice. The difference in the number of hepatic lymphocytes transferred reflected the increased recovery of these cells in the immunized mice. We took this strategy because we considered it to reflect the immune competence of each group of mice more accurately than transferring a fixed number of cells. Two days after the adoptive transfer, recipient mice were challenged with 1,000 sporozoites to determine the protective effect of the transferred cells against EE forms of parasites (Fig. 6A). The parasite burden of mice that received immune serum showed no significant difference from that of naive mice or the recipients of unprimed lymphocytes. In contrast, the parasite burdens in the recipients of cells from the immunized mice were lower than those in control groups. In particular, the level of parasite burden in mice that received immune hepatic lymphocytes was statistically significant.

Whole spleen cells and hepatic lymphocytes were used for our adoptive transfer experiment. We considered it necessary to rule out the possible contribution of anti-MSP1 Ab produced by the transferred B cells. Therefore, we determined the levels of anti-MSP1 Ab in the sera of the recipient mice. Serum was collected from each mouse 2 days after the infection, and levels of Ab specific for MSP1 were determined by ELISA (Fig. 6B). No anti-MSP1 Ab was detected in the serum of mice transferred with immune lymphocytes, while high levels of Ab were detected in the sera of mice that received immune serum. These results indicated that the protective immunity against EE forms of *P. yoelii* induced by immunization with MSP1-hsc70 was mediated by cellular and not humoral immunity.

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 *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^a), C3H (H-2^k), and BALB/c (H-2^d). Two weeks after the third immunization, mice were challenged with *P. yoelii* sporozoites and parasitemia was monitored daily. A/J mice were relatively resistant to *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

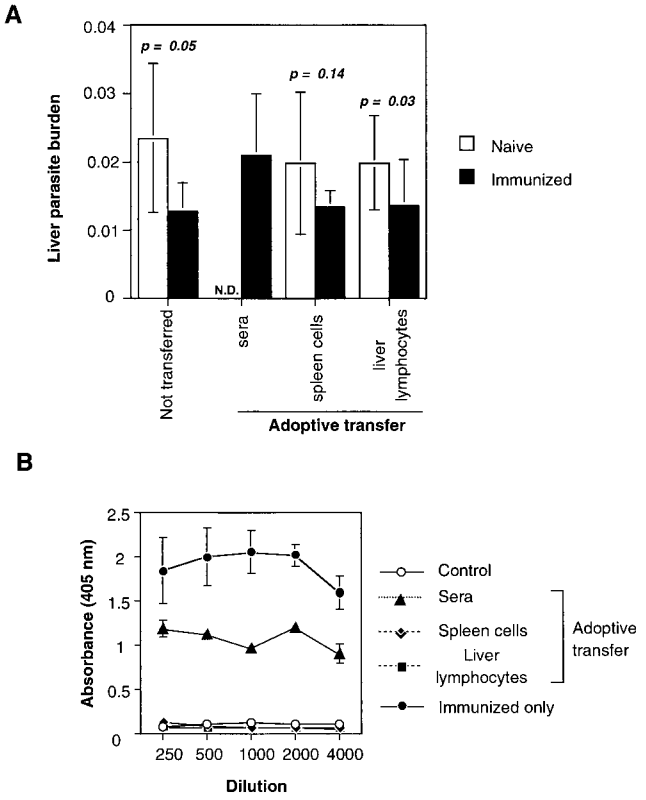

FIG. 6. Adoptive transfer of lymphocytes and antiserum from C57BL/6 mice immunized with MSP1-hsc70 into naive recipient mice. (A) C57BL/6 mice not immunized or immunized with MSP1-hsc70 seven times were used as donors. Two weeks after the last immunization, spleen cells and hepatic lymphocytes were collected 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 appear 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-

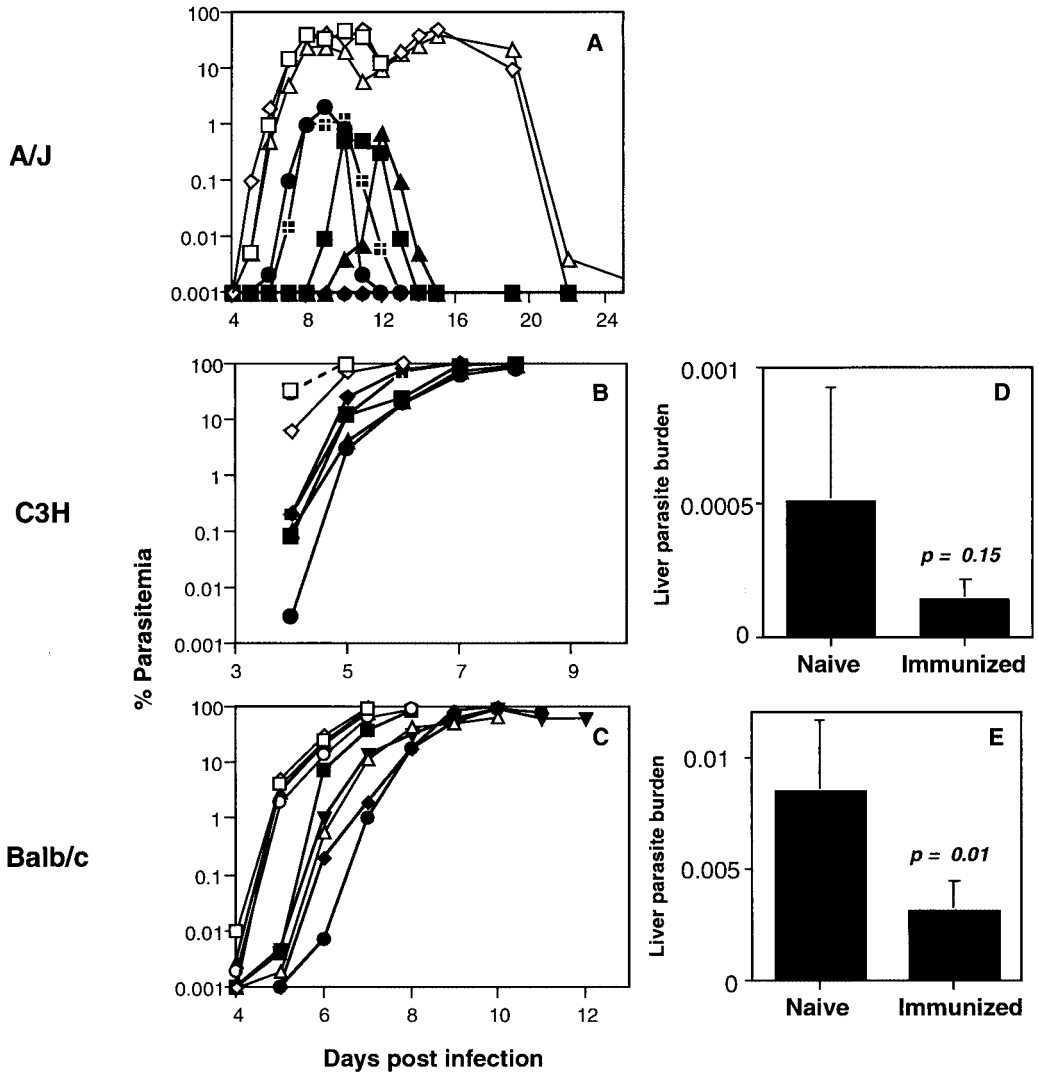

FIG. 7. The course of parasitemia in A/J (A), C3H (B), and BALB/c (C) mice and liver parasite burden in C3H (D) and BALB/c (E) mice that were immunized with MSP1-hsc70 and challenged with *P. yoelii* sporozoites. (A to C) Mice not immunized (open symbols) or immunized with MSP1-hsc70 three times (filled symbols) were challenged with 50 *P. yoelii* sporozoites i.v. 2 weeks after the last immunization. The course of parasitemia in each mouse was monitored daily after day 4 of the infection by microscopic examination of the stained blood smears. The data show parasitemia for five individual mice in each group, and represent two experiments with similar results. (D and E) C3H and BALB/c mice (five mice/group) were not immunized (control) or immunized four times with MSP1-hsc70 and challenged with 1,000 sporozoites i.v. The levels of liver parasite burden were determined by real time RT-PCR as described in the legend for Fig. 5.

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 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.

ACKNOWLEDGMENTS

We thank M. Torii for *P. yoelii 17XL* and *A. stephensi* isolates and S. Matsumoto for MSP1 cDNA and anti-MSP1 Ab. We also thank M. Torii, K. Arisawa, H. Watanabe, and S. Matsumoto for suggestions and help and G. Massey for editorial assistance with the manuscript.

This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan.

REFERENCES

1. **Aley, S. B., J. W. Barnwell, M. D. Bates, W. E. Collins, and M. R. Hollingdale.** 1987. Exoerythrocytic schizonts recognized by monoclonal antibodies against blood-stage schizonts. Exp. Parasitol. **64:**188–194.
2. **Becker, S. I., R. Wang, R. C. Hedstrom, J. C. Aguiar, T. R. Jones, S. L. Hoffman, and M. J. Gardner.** 1998. Protection of mice against *Plasmodium yoelii* sporozoite challenge with *P. yoelii* merozoite surface protein 1 DNA vaccines. Infect. Immun. **66:**3457–3461.
3. **Blackman, M. J., H. G. Heidrich, S. Donachie, J. S. McBride, and A. A. Holder.** 1990. A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. J. Exp. Med. **172:**379–382.
4. **Briones, M. R., M. Tsuji, and V. Nussenzweig.** 1996. The large difference in infectivity for mice of *Plasmodium berghei* and *Plasmodium yoelii* sporozoites cannot be correlated with their ability to enter into hepatocytes. Mol. Biochem Parasitol. **77:**7–17.
5. **Chitarra, V., I. Holm, G. A. Bentley, S. Petres, and S. Longacre.** 1999. The crystal structure of C-terminal merozoite surface protein 1 at 1.8 Å resolution, a highly protective malaria vaccine candidate. Mol. Cell **3:**457–464.
6. **Daly, T. M., and C. A. Long.** 1993. A recombinant 15-kilodalton carboxyl-terminal fragment of *Plasmodium yoelii yoelii* 17XL merozoite surface protein 1 induces a protective immune response in mice. Infect. Immun. **61:**2462–2467.
7. **Goossens, P. L., H. Jouin, G. Marchal, and G. Milon.** 1990. Isolation and flow cytometric analysis of the free lymphomyeloid cells present in murine liver. J. Immunol. Methods **132:**137–144.
8. **Hirunpetcharat, C., P. Vukovic, X. Q. Liu, D. C. Kaslow, L. H. Miller, and M. F. Good.** 1999. Absolute requirement for an active immune response involving B cells and Th cells in immunity to *Plasmodium yoelii* passively acquired with antibodies to the 19-kDa carboxyl-terminal fragment of merozoite surface protein-1. J. Immunol. **162:**7309–7314.
9. **Holder, A. A., and R. R. Freeman.** 1981. Immunization against blood-stage rodent malaria using purified parasite antigen. Nature **294:**361–364.
10. **Huang, Q., J. F. Richmond, K. Suzue, H. N. Eisen, and R. A. Young.** 2000. In vivo cytotoxic T lymphocyte elicitation by mycobacterial heat shock protein 70 fusion proteins maps to a discrete domain and is $CD4^+$ T cell independent. J. Exp. Med. **191:**403–408.
11. **Kang, Y., P. A. Calvo, T. M. Daly, and C. A. Long.** 1998. Comparison of humoral immune responses elicited by DNA and protein vaccines based on merozoite surface protein-1 from *Plasmodium yoelii*, a rodent malaria parasite. J. Immunol. **161:**4211–4219.
12. **Matsumoto, S., H. Yukitake, H. Kanbara, and T. Yamada.** 1998. Recombinant *Mycobacterium bovis* bacillus Calmette-Guerin secreting merozoite surface protein 1 (MSP1) induces protection against rodent malaria parasite infection depending on MSP1-stimulated interferon gamma and parasite-specific antibodies. J. Exp. Med. **188:**845–854.
13. **Murphy, J. R., S. Baqar, J. R. Davis, D. A. Herrington, and D. F. Clyde.** 1989. Evidence for a 6.5-day minimum exoerythrocytic cycle for *Plasmodium falciparum* in humans and confirmation that immunization with a synthetic peptide representative of a region of the circumsporozoite protein retards infection. J. Clin. Microbiol. **27:**1434–1437.
14. **Nardin, E. H., and R. S. Nussenzweig.** 1993. T cell responses to pre-erythrocytic stages of malaria: role in protection and vaccine development against pre-erythrocytic stages. Annu. Rev. Immunol. **11:**687–727.
15. **Nussenzweig, R. S., J. Vanderberg, H. Most, and C. Orton.** 1967. Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. Nature **216:**160–162.
16. **Renia, L., I. T. Ling, M. Marussig, F. Miltgen, A. A. Holder, and D. Mazier.** 1997. 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. Infect. Immun. **65:**4419–4423.
17. **Schofield, L., J. Villaquiran, A. Ferreira, H. Schellekens, R. Nussenzweig, and V. Nussenzweig.** 1987. Gamma interferon, $CD8^+$ T cells and antibodies required for immunity to malaria sporozoites. Nature **330:**664–666.
18. **Srivastava, P. K., A. Menoret, S. Basu, R. J. Binder, and K. L. McQuade.** 1998. Heat shock proteins come of age: primitive functions acquire new roles in an adaptive world. Immunity **8:**657–665.
19. **Suhrbier, A., A. A. Holder, M. F. Wiser, J. Nicholas, and R. E. Sinden.** 1989. Expression of the precursor of the major merozoite surface antigens during the hepatic stage of malaria. Am. J. Trop. Med. Hyg. **40:**351–355.
20. **Szarfman, A., D. Walliker, J. S. McBride, J. A. Lyon, I. A. Quakyi, and R. Carter.** 1988. Allelic forms of gp195, a major blood-stage antigen of *Plasmodium falciparum*, are expressed in liver stages. J. Exp. Med. **167:**231–236.
21. **Taguchi, T., J. R. McGhee, R. L. Coffman, K. W. Beagley, J. H. Eldridge, K. Takatsu, and H. Kiyono.** 1990. Detection of individual mouse splenic T cells producing IFN-gamma and IL-5 using the enzyme-linked immunospot (ELISPOT) assay. J. Immunol. Methods **128:**65–73.
22. **Tian, J. H., L. H. Miller, D. C. Kaslow, J. Ahlers, M. F. Good, D. W. Alling, J. A. Berzofsky, and S. Kumar.** 1996. Genetic regulation of protective immune response in congenic strains of mice vaccinated with a subunit malaria vaccine. J. Immunol. **157:**1176–1183.
23. **Tsuji, M., P. Romero, R. S. Nussenzweig, and F. Zavala.** 1990. $CD4^+$ cytolytic T cell clone confers protection against murine malaria. J. Exp. Med. **172:**1353–1357.
24. **Udono, H., T. Yamano, Y. Kawabata, M. Ueda, and K. Yui.** 2001. Generation of cytotoxic T lymphocytes by MHC class I ligands fused to heat shock cognate protein 70 (hsc70). Int. Immunol. **13:**1233–1242.
25. **Watanabe, H., K. Ohtsuka, M. Kimura, Y. Ikarashi, K. Ohmori, A. Kusumi, T. Ohteki, S. Seki, and T. Abo.** 1992. Details of an isolation method for hepatic lymphocytes in mice. J. Immunol. Methods **146:**145–154.
26. **Weiss, W. R., M. F. Good, M. R. Hollingdale, L. H. Miller, and J. A. Berzofsky.** 1989. Genetic control of immunity to *Plasmodium yoelii* sporozoites. J. Immunol. **143:**4263–4266.

Editor: J. M. Mansfield