Recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin Secreting Merozoite Surface Protein 1 (MSP1) Induces Protection against Rodent Malaria Parasite Infection Depending on MSP1-stimulated Interferon γ and Parasite-specific Antibodies

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Summary

The merozoite surface protein 1 (MSP1) has emerged as a leading malaria vaccine candidate at the erythrocytic stage. Recombinant bacillus Calmette-Guérin (rBCG), which expressed a COOH-terminal 15-kD fragment of MSP1 of *Plasmodium yoelii* (MSP1-15) as a fusion protein with a secretory protein of *Mycobacterium kansasii*, was constructed. Immunization of mice with this rBCG induced a higher degree of protection against blood-stage parasite infection than with recombinant MSP1-15 in the RIBI adjuvant (RIBI ImmunoChem Research, Inc., Hamilton, MT) or incomplete Freund's adjuvant systems. We studied the mechanism of protection induced by MSP1-15, and found that interferon (IFN)-γ had a major role in protection in all adjuvant systems we examined. Mice that produced low amounts of MSP1-15 stimulated IFN-γ and could not control parasite infection. The antibody against MSP1-15 did not play a major role in protection in this system. After parasite infection, immunoglobulin G2a antibodies, which had been produced by IFN-γ stimulation, were induced and subsequently played an important role in eradicating parasites. Thus, both cellular and humoral immune responses were essential for protection from malaria disease. These data revealed that BCG is a powerful adjuvant to induce such a protective immune response against malaria parasites.

Key words: malaria vaccine • recombinant bacillus Calmette-Guérin • cytokine • protection • secretion system

Malaria is still a serious problem in tropical countries. 500 million people suffer from malaria, and deaths total 2.7 million a year (1). The main hope for controlling this disease is to develop effective vaccines. Several protective antigens have been identified, and analysis of epitopes in each of them has been explored (1). However, to promote and safely prolong the immune response, the development of effective adjuvants is required.

Widespread use of the Calmette-Guérin bacillus (BCG)[1] has demonstrated some advantages, such as excellent immune adjuvant activity, long-persisting effects, safety, and low cost, leading us to use it as a vaccine vehicle for delivering foreign antigens. A transformation system for mycobacteria enabled us to express a foreign gene in BCG (2). With this system, we used the α antigen of *Mycobacterium kansasii* (α-k) to secrete a foreign antigen from BCG (3). By using a promoter from heat shock protein, two groups succeeded in expressing gag, pol, and env of HIV-1 in BCG and induced humoral and cellular responses in immunized mice (4, 5). Subsequently, varieties of the expression system have also been reported (6–12). In recent years, protective humoral responses against *Borrelia burgdorferi* (13, 14) and *Streptococcus pneumoniae* (15) were efficiently induced using the recombinant (r)BCG system. This efficacy was seen only when the protective antigens were secreted from BCG.

Arguments supporting BCG as a suitable vector for a malaria vaccine have been raised. First, BCG immunization alone could provide nonspecific resistance to malaria infection in mice (16, 17). Second, the use of a tuberculin puri-

[1]*Abbreviations used in this paper:* BBS, borate-buffered saline; BCG, bacillus Calmette-Guérin; CF, culture filtrates; D-NMMA, N^G-monomethyl-d-arginine; GST, glutathione *S*-transferase; HSBBS, BBS containing 0.5 M NaCl; α-k, α antigen of *Mycobacterium kansasii*; L-NMMA, N^G-monomethyl-l-arginine; MBP, maltose binding protein; MSP1, merozoite surface protein 1; MSP1-15, COOH-terminal fragment of MSP1 from *Plasmodium yoelii* 17XL; MSP1-19, COOH-terminal 19-kD fragment of MSP1 from *Plasmodium falciparum*; NO, nitric oxide; PAb, polyclonal antibody; PBS-T80, PBS containing 0.1% Tween 80; RAS, RIBI adjuvant system; rBCG, recombinant BCG clone; rBCGMSP1-15, rBCG secreting α-k–MSP1-15.

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fied protein derivative–parasite antigen conjugate and live BCG priming induced protection against a malaria parasite without strong adjuvants (18). Therefore, the rBCG system is expected to be an excellent system for malaria vaccine development. Trials have been progressing, though a successful result has not yet been obtained (10, 19).

The merozoite surface protein 1 (MSP1) is one of the leading vaccine candidates at the erythrocytic stage. This molecule has been identified in almost all of the *Plasmodium* species that infect humans (20, 21), simians (22, 23), and rodents (24–26). Molecular mass ranges from 185 to 250 kD. Protective immunity induced by vaccination with MSP1 was demonstrated initially in the *Plasmodium yoelii* model (24). Subsequently, this finding was also confirmed in a nonhuman primate model using MSP1 from *Plasmodium falciparum*, the most virulent human malaria parasite (27–34). MSP1 is proteolytically cleaved into several fragments by two processing steps (35–39). Only a COOH-terminal 19-kD fragment of MSP1 (MSP1-19) remains on the merozoite surface during invasion into a new erythrocyte (35). The COOH-terminal fragment contains a series of cysteine residues that are conserved among various *Plasmodium* species. Structural examination showed that it possessed two epidermal growth factor–like domains (36). Antibodies specific to MSP1-19 inhibited invasion of merozoites into erythrocytes in vitro (35, 40, 41). Some MSP1-19–specific antibodies inhibited the protease-mediated secondary processing of MSP1 (42). In rodent models, an mAb which protected mice against infection in passive transfer experiments recognized MSP1-15 (43–45). It has been reported that immunization with MSP1-15 from *P. yoelii* can protect mice against lethal infection (46). In light of the reports described above, the COOH-terminal fragment of MSP1 should be an attractive component of a subunit vaccine against malaria. Therefore, the combination of the COOH-terminal polypeptide and BCG was expected to be a powerful tool for developing an effective malaria vaccine.

In this study, we first constructed rBCG secreting MSP1-15 as a fusion protein with α-k (α-k–MSP1-15), and found that rBCG could induce significant protection against a *P. yoelii* challenge in immunized C3H/He mice. This system was much more efficient than other artificial adjuvants for MSP1-15 in C3H/He mice. We studied extensively the mechanism of protection in immunized mice, and found that IFN-γ played an important role in this protection. Antibodies against the parasite, induced in the course of infection, also eventually contributed to the subsequent protection.

Materials and Methods

Plasmids and Bacterial Strains. BCG Tokyo was used as a host for plasmid pSO246 (47) and its derivatives. BCG Tokyo and its transformants were grown in Middlebrook 7H9 broth (Difco Laboratories, Inc., Detroit, MI) supplemented with 10% albumin-dextrose-catalase (ADC) enrichment (Difco Laboratories, Inc.) and 0.5% Tween 80 (7H9 ADC medium). rBCG was selected by growing on Middlebrook 7H10 agar (Difco Laboratories, Inc.) containing 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco Laboratories, Inc.), 0.5% glycerol, 400 U/ml penicillin, and 100 μg/ml cycloheximide (7H10 OADC agar). *Escherichia coli* strain XL1-blue was used as a host for plasmid pGEX2T (Pharmacia Biotech, Inc., Uppsala, Sweden), pMALC2 (New England Biolabs, Inc., Beverly, MA), pBluescript SK(+) (Stratagene, La Jolla, CA), and their derivatives. The *E. coli* strain was grown in TY broth with or without 2% glucose.

Animals. C3H/He and A/J female mice were purchased from Japan SLC (Hamamatsu, Japan). C57BL/6 female mice were purchased from Charles River Laboratories (Wilmington, MA).

Construction of Expression Vectors to Secrete MSP1-15 from BCG. An MSP1-15 gene segment (amino acids 1618–1722 [48]) was amplified by PCR targeted to the genomic DNA of *P. yoelii* 17XL. Primers used to amplify the MSP1-15 gene were primer A (for the sense strand), 5′-CCctcgagCATAGCCTCAATAGCT, and primer B (for the antisense strand), 5′-CCctcgagCCCATAAAGCTGGAAG. The added sequence indicated by small letters refers to sites recognized by restriction enzymes. pKH20, which included an α-k gene (49), was then digested with both BamH1 and HindIII. The 2-kbp BamH1-HindIII fragment containing the α-k gene was inserted into the same sites of pBluescript SK (+). This plasmid was designated pBSSKH20. The DNA fragment amplified with primers A and B was digested with Xho1 and inserted into the same site of pKH20. This plasmid was designated pUCMSP1-15. It was then digested with BamHI and HindIII. The 2.4-kbp fragment containing an α-k–MSP1-15 hybrid gene was inserted into the same sites of pSO246 (47). The final construct was designated pSOMSP1-15 (for the construction map, see Fig. 1 *A*). It was transformed into BCG Tokyo by electroporation as described previously (19).

Expression of MSP1-15 by Two Systems in E. coli. To prepare the DNA encoding recombinant fusion protein of *Schistosoma japonicum* glutathione *S*-transferase (GST) and MSP1-15 (GST–MSP1-15), the oligonucleotides 5′-GGggatccCACATAGCCTCAATAGCT (for the sense strand) and 5′-CCCgaattcTCCCATAAAGCTGGAAG (for the antisense strand) were synthesized. PCR was performed using the above-described primers targeting genomic DNA from *P. yoelii* 17XL. Amplified DNA was digested with both BamHI and EcoRI. It was then inserted into the same site of pGEX2T, and the final construct was transformed into *E. coli*. Next, to prepare the DNA encoding the recombinant fusion protein of maltose binding protein (MBP) and MSP1-15 (MBP–MSP1-15), the oligonucleotides 5′-GGGgaattcCACATAGCCTCAATAGCT (for the sense strand) and 5′-GGGctgcagTCCCATAAAGCTGGAAG (for the antisense strand) were synthesized. The DNA amplified using the above-described primers was digested with both EcoR1 and PstI. It was then inserted into the same site of pMALC2, and transformed into *E. coli*. GST–MSP1-15 and MBP–MSP1-15 were isolated from bacterial lysates by affinity chromatography according to the manufacturer's instructions (Pharmacia Biotech, Inc., and New England Biolabs, Inc.).

Polyclonal Antibody Preparation. The polyclonal antibody (PAb) against *P. yoelii* 17XL was prepared from mice which were repeatedly infected with *P. yoelii* 17XL. The rabbit anti–α-k PAb was provided by Dr. Matsuo (Central Research Laboratories, Ajinomoto Co. Inc., Kawasaki, Japan [49]).

Immunization of C3H/He Mice with rBCG. C3H/He mice at 7–10 wk of age were immunized intravenously with 10^6 CFU of BCG transformed with pSOMSP1-15 (rBCGMSP1-15) in 200 μl of PBS containing 0.1% Tween 80 (PBS-T80). A control group of mice was injected with 10^6 CFU of BCG in 200 μl of PBS-

T80 or PBS-T80 only. After 30 d, the same amount of each sample was injected intraperitoneally to boost the immune response. Before 1 wk from parasite challenge, sera were collected from the eye veins of immunized mice and pooled at −80°C until use.

Immunization with GST–MSP1-15 in Artificial Adjuvants. Each kind of mouse was immunized with 10 μg i.p. of GST–MSP1-15 in either IFA or the Rivi adjuvant system (RAS). The control group was injected with 10 μg of GST in the same way. 3 wk after the first vaccination, a second immunization was carried out with the same amounts of each sample.

Measurement of Antibody Titer against MSP1-15 and Blood-stage Parasite. The titer of whole immunoglobulins (Igs), IgE, IgM, IgG1, IgG2a, IgG2b, and IgG3 antibodies against MSP1-15 and *P. yoelii* lysate was measured by ELISA as follows. Each well of a microtiter plate was coated with 10 μg/ml of MBP–MSP1-15 or *P. yoelii* lysate (blood-stage) in 100 μl of borate-buffered saline (BBS: 167 mM borate, 134 mM NaCl, pH 8.0) by incubation at room temperature for 2 h. The wells were washed once with BBS by shaking on the automatic ELISA washer. The wells were then blocked with 200 μl of 3% BSA in BBS for 1 h at room temperature and washed with BBS containing 0.5 M NaCl (HS-BBS). 100 μl of each serum diluted to 1:100 with BBS containing 1% BSA was added to individual wells and incubated at 37°C for 30 min. After washing with HSBBS seven times, each well was filled with 100 μl of peroxidase-conjugated anti–mouse Ig, IgM, IgE, IgG1, IgG2a, IgG2b, or IgG3 (Organon Teknika-Cappel, Durham, NC) antibodies diluted to 1:1,000 with BBS containing 1% BSA, and incubated at 37°C for 30 min. After washing with HSBBS seven times and once with BBS, 100 μl of a solution of 0.4 mg/ml of *o*-phenylenediamine dihydrochloride in 80 mM citrate-phosphate, pH 5.0, was added to the wells. OD was measured at 492 nm.

Challenge Infection of P. yoelii 17XL. All mice were challenged with 10^4 *P. yoelii* 17XL–parasitized erythrocytes intravenously or intraperitoneally 1 mo after the final immunization. The course of infection was monitored by microscopic examination of tail-blood smears stained with Giemsa.

Immune Sera Passive Transfer Experiment. Sera were collected from mice immunized with rBCGMSP1-15 or GST-MSP1-15 in RAS at 1 wk before or 30 d after parasite challenge. A group of naive C3H/He mice was given 200 μl of serum intraperitoneally. 1 h later, mice were challenged with 10^4 *P. yoelii* 17XL–parasitized erythrocytes intraperitoneally.

Measurement of Cytokine Production. 1 mo after the final immunization, mice were killed, and spleens were removed from at least three mice of each group. Red cells were lysed by Tris–ammonium buffer and washed with RPMI 1640 medium. Spleen cells were suspended in RPMI 1640 medium with 10% FCS containing 5 μg/ml of MBP or MBP–MSP1-15 at a concentration of 10^6/ml, and 200 μl of suspension was put into a round-bottomed well (Iwaki Glass Co., Ltd., Funabashi, Japan). The plate was incubated at 37°C for 48 h, and supernatants were then collected and analyzed. Measurement of IFN-γ and IL-4 was carried out using ELISA kits (Genzyme Corp., Cambridge, MA) as described in the kit instructions.

Neutralization of IFN-γ. For neutralization of IFN-γ, mice were treated with 100 μg i.p. of the rat anti–mouse IFN-γ mAb (Endogen, Inc., Cambridge, MA) 2 d before, the day of, and 2 d after the challenge infection. As controls, mice were treated with rat IgG (Endogen, Inc.) in the same way.

Treatment of Immunized Mice with Inhibitor of Nitric Oxide Synthase. Mice immunized with rBCGMSP1-15 were injected with 250 mg/kg/d i.p. of N^G-monomethyl-l-arginine (L-NMMA) every day from 2 d before to 2 d after infection. As controls, mice were treated with N^G-monomethyl-d-arginine (D-NMMA) in the same way.

Results

Secretion of Conformational MSP1-15 from rBCG as a Fusion Protein with α-k. BCG was transformed with pSOMSP1-15 (Fig. 1 *A*), and designated rBCGMSP1-15. The rBCGMSP1-15 was cultured for 3 wk on Sauton medium (3). After precipitation of proteins in culture filtrates (CF) from rBCGMSP1-15 with 80% NH_4SO_4, 20 μg of the proteins was applied to SDS-PAGE (Fig. 1 *B*). The proteins in the gels were visualized by staining the gel with Coomassie brilliant blue (Fig. 1 *B*). The additional bands at 40 and 22 kD (lane *3*) were estimated to be α-k–MSP1-15 and its degraded product because both anti–α-k and anti-GST–MSP1-15 PAbs reacted to these additional bands (data not shown). It was reported that the native structure of MSP1 is important to induce protection (41). Therefore, we assessed whether antibodies against native MSP1-15 reacted to α-k–MSP1-15. After native PAGE, the proteins were blotted and reacted with the PAb against blood-stage *P. yoelii* 17XL. The results are presented in Fig. 1 *C*. A strong reaction to the naive sample including α-k–MSP1-15 was observed (lane *4*),

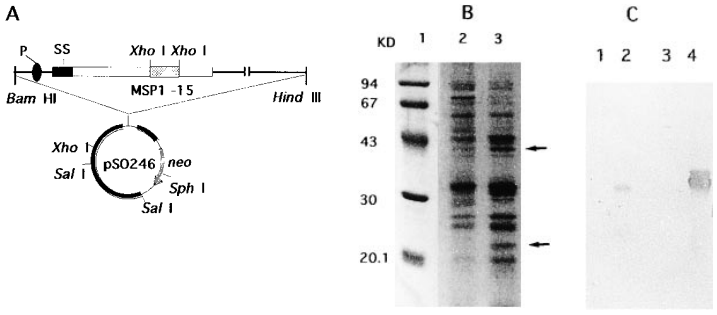

Figure 1. Construction of pSOMSP1-15 (*A*) and detection of secreted α-k–MSP1-15 from rBCG by SDS-PAGE (*B*) and Western blot analysis (*C*). (*A*) Plasmid map of pSOMSP1-15. *P*, A promoter of the α-k gene. *SS*, An encoding region of the signal sequence. *neo*, A neomycin phosphotransferase gene. *Striped bar*, MSP1-15 gene. *White bar*, An α-k structural gene. The experimental procedures are described in Materials and Methods. (*B*) Coomassie brilliant blue–stained gels, resolved by SDS-PAGE. Lanes are as follows: molecular mass marker (lane *1*), 20 μg of secreted proteins from BCG transformed with empty plasmid (lane *2*), and BCG transformed with pSOMSP1-15 (lane *3*). *Arrows*, Additional bands estimated to be α-k–MSP1-15 and its degraded product. (*C*) Western blot analysis by anti–blood-stage *P. yoelii* antibodies. CF from BCG transformed with empty plasmid (lanes *1* and *3*) and from BCG transformed with pSOMSP1-15 (lanes *2* and *4*) were suspended in sample buffer without SDS and 2-ME. The samples in lanes *1* and *2* were heat-denatured before PAGE. The samples were subjected to native PAGE and transferred to polyvinylidene difluoride membrane. The membrane was reacted with anti–blood-stage *P. yoelii* sera.

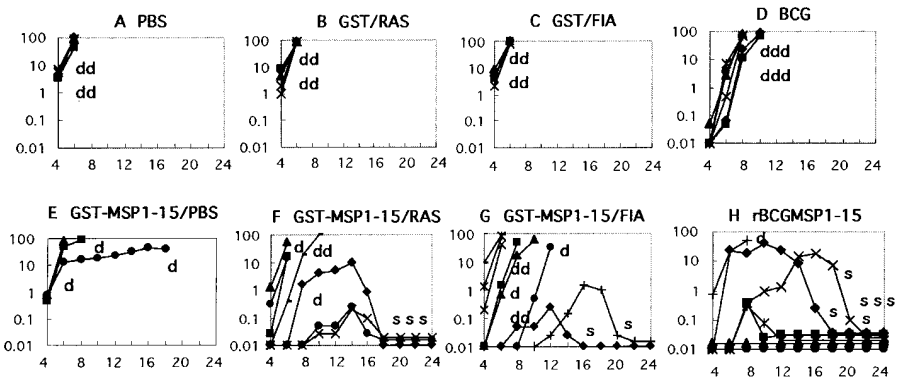

Figure 2. Course of infection of *P. yoelii* in individual female C3H/He mice. Mice were immunized with PBS (*A*), GST in RAS (*B*), GST in IFA (*FIA*; *C*), parental BCG in PBS-T80 (*D*), GST–MSP1-15 in PBS (*E*), GST–MSP1-15 in RAS (*F*), GST–MSP1-15 in IFA (*FIA*; *G*), and rBCGMSP1-15 in PBS-T80 (*H*). The immunization procedure is described in Materials and Methods. PBS-T80 gave results similar to PBS immunization. *y-axis*, Percentage of infected erythrocytes. Parasitemia was estimated from Giemsa-stained smears. *x-axis*, Days from challenge. Each line shows the course of parasitemia of an individual mouse. *d*, Dead mouse. *s*, Self-cured mouse. These experiments were repeated at least three times.

but reaction to the heat-denatured sample was diminished (lane *2*). These results indicated that (*a*) almost all of the antibodies against MSP1-15 induced by infection with *P. yoelii* recognized conformational epitopes in MSP1-15; and (*b*) α-k–MSP1-15 secreted from rBCGMSP1-15 formed a similar antigenic conformation as the native MSP1-15. GST–MSP1-15 purified from recombinant *E. coli* was also recognized by antibody against conformational determinants in MSP1-15, as described previously (46), as was MBP–MSP1-15 (data not shown).

Evaluation of the Protective Efficacy Induced by MSP1-15 in Several Adjuvants. All of the C3H/He mice immunized with samples were challenged with 10^4 *P. yoelii* 17XL, and the levels of protection of various adjuvants were compared. All mice immunized with PBS (Fig. 2 *A*), GST in RAS (Fig. 2 *B*), and GST in IFA (Fig. 2 *C*) showed detectable parasitemia 4 d after challenge, as expected (Fig. 2, *A–C*). PBS-T80 gave results similar to PBS immunization (data not shown). These mice developed fulminating infection with high parasitemia leading to death. When mice were immunized with live BCG, it was of interest to see a 2–4-d delay of the onset of parasitemia, though they eventually developed fulminating infection and died (Fig. 2 *D*). Results similar to BCG immunization were obtained when the mice were immunized with rBCG-transformed empty plasmids or plasmids containing only the α-k gene (data not shown). Slight preventive effects on parasitemia were observed when mice were immunized with GST–MSP1-15 without adjuvant (Fig. 2 *E*). Combinations of a variety of adjuvants stimulated protection by MSP1-15 (Fig. 2, *F–H*). Three out of seven mice immunized with GST–MSP1-15 in RAS (Fig. 2 *F*), two out of eight mice immunized with GST–MSP1-15 in IFA (Fig. 2 *G*), and six out of seven mice immunized with rBCGMSP1-15 (Fig. 2 *H*) survived the infection. These data indicate that three of the adjuvants examined in this study are effective for vaccination with MSP1-15, though their levels of efficacy are different. The rBCG system was the most effective.

IFN-γ versus IL-4 Production in Spleens from Immunized Mice. That the spleen plays an important role in host defense against *Plasmodium* infection is well-established. We examined the production of IFN-γ as representative of Th1-type responses and of IL-4 as representative of Th2-type responses in spleen cells. 30 d after the final immunization, spleens were removed from mice immunized with rBCGMSP1-15. The cells were then cultured with MBP–MSP1-15. As controls, cells were cultured with MBP alone or medium only. After incubation for 48 h, the cytokines produced in the culture medium were assessed by ELISA, and the results are presented in Fig. 3. An appreciable amount of IFN-γ, but not IL-4, was detected. Similar results were obtained using spleen cells from mice immunized with GST–MSP1-15 in IFA (data not shown). These data suggested that MSP1-15 induced IFN-γ but not IL-4 in C3H/He mice regardless of the adjuvant used.

IFN-γ and Nitrous Oxide Play Important Roles in Clearance of Infection. Next, we assessed the role of IFN-γ in protection against parasite infection. C3H/He mice were immunized with rBCGMSP1-15 and treated with IFN-γ neutralizing antibodies. The control mice were treated with the same dose of rat IgG. The time courses of para-

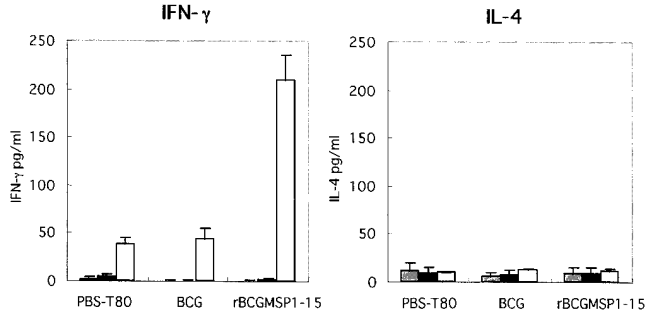

Figure 3. Production of IFN-γ and IL-4 in spleen cells stimulated with control saline (*gray bar*), MBP (*black bar*), and MBP–MSP1-15 (*white bar*). *y-axis*, Amounts of produced cytokines in the supernatants. Immunizing samples are indicated below the x-axis. Measurements of IFN-γ and IL-4 were made with the ELISA kit purchased from Genzyme Corp. (Cambridge, MA). The amounts of cytokines were calculated using serial dilutions of supernatants to obtain values that fell within the linear range of the standard curve. Data are expressed as the mean ± SD for three cultures.

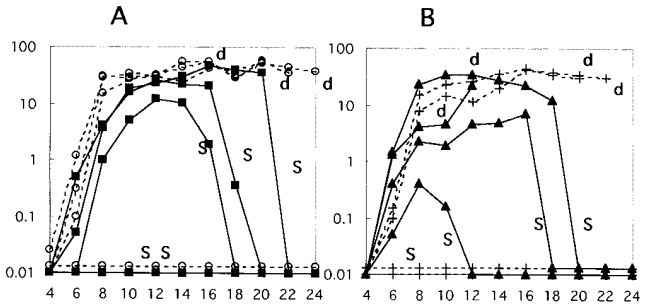

Figure 4. (*A*) Course of infection of *P. yoelii* in C3H/He mice immunized with rBCGMSP1-15 and treated with either control rat IgG (*filled square*) or anti–IFN-γ neutralizing antibody (*open circle*). (*B*) Similar experiments were carried out with either D-NMMA (*filled triangle*) or L-NMMA (+). Each line shows the course of parasitemia of an individual mouse. *y-axis*, Percentage of parasite-infected erythrocytes. *x-axis*, Days after infection. *d*, Dead mouse. *s*, Self-cured mouse.

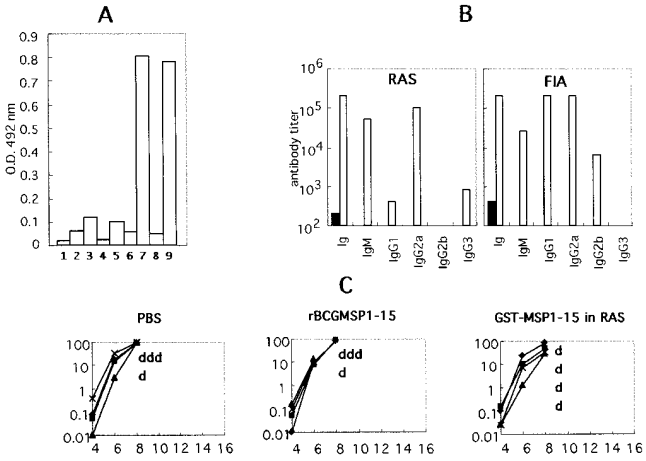

Figure 5. Analysis of sera before challenge. (*A*) Level of MSP1-15–specific antibody in sera from mice immunized with each sample before challenge. Lanes are as follows: *1*, PBS-T80; *2*, BCG in PBS-T80; *3*, rBCGMSP1-15 in PBS-T80; *4*, PBS; *5*, GST–MSP1-15 in PBS; *6*, GST in RAS; *7*, GST–MSP1-15 in RAS; *8*, GST in IFA; *9*, GST–MSP1-15 in IFA. *y-axis*, OD at 492 nm, after subtracting OD of the control sample. Sera were diluted to 1:100. (*B*) Isotype distribution of antibodies against MBP–MSP1-15. Serum antibodies were obtained before challenge from mice immunized with GST (*black bar*) or GST–MSP1-15 (*white bar*) in RAS (*left*) or IFA (*FIA*; *right*). The level of Igs against MBP–MSP1-15 and their distribution into IgM, IgG1, IgG2a, IgG2b, and IgG3 subclasses were assessed by ELISA using anti–mouse subclass-specific antibodies. (*C*) Passive immunity imparted by transfer of serum from mice immunized with PBS (*left*), rBCGMSP1-15 (*middle*), and GST–MSP1-15 in RAS (*right*). 1 h after passive transfer of 0.2 ml of each serum, mice were challenged with 10^4 *P. yoelii*–parasitized erythrocytes intraperitoneally. Each line shows the course of parasitemia of an individual mouse. *y-axis*, Percentage of parasitemia. *x-axis*, Days from infection.

sitemia in these experiments are presented in Fig. 4 *A*. Three out of four mice in both groups developed infection. As expected, all of the control mice cleared the infection and were self-cured. On the other hand, three out of the four mice treated with IFN-γ neutralizing antibodies eventually died, although an obvious difference in parasitemia from the control group was not observed at the beginning of the infection (Fig. 4 *A*). It is well known that IFN-γ enhances the production of nitrous oxide (NO), which is effective against intracellular parasites. We also assessed the role of NO by neutralization with the inhibitor of NO synthase, L-NMMA, in vivo. Mice were immunized with rBCGMSP1-15 and treated with L-NMMA intraperitoneally. As shown in Fig. 4 *B*, three out of four control mice treated with D-NMMA were self-cured of disease, whereas three out of four mice treated with L-NMMA could not control the infection and eventually died. These data indicate that both IFN-γ and NO have important roles in protection against infection induced by rBCGMSP1-15 immunization.

Evaluation of the Protectiveness of the Sera before Parasite Challenge. The humoral immune response to MSP1-15 and *P. yoelii* lysate was monitored by ELISA. No obvious antibody specific to MSP1-15 was observed in mice immunized with rBCGMSP1-15 before parasite challenge (Fig. 5 *A*, lane *3*). In contrast, a high titer of MSP1-15–specific antibody was observed in the sera of mice immunized with GST–MSP1-15 in both RAS and IFA before challenge (Fig. 5 *A*, lanes *7* and *9*). Assessing the isotypes of these anti–MSP1-15–specific Igs revealed that RAS stimulated MSP1-15–specific IgM and IgG2a dominantly (Fig. 5 *B*, *left*) and IFA stimulated IgM, IgG1, IgG2a, and IgG2b (Fig. 5 *B*, *right*). We also assessed the protective efficacy of the sera before challenge. Naive C3H/He mice were given 0.2 ml i.p. of sera from mice before parasite challenge. 1 h later, 10^4 *P. yoelii*–infected erythrocytes were injected intraperitoneally. The results showed that sera before challenge were not protective (Fig. 5 *C*), though the sera included a high titer of MSP1-15–specific antibody (Fig. 5 *A*). This suggested that the MSP1-15–specific antibody was not an essential factor in the protection induced by immunization with both rBCGMSP1-15 and GST–MSP1-15 in artificial adjuvants.

Evaluation of the Protectiveness of the Sera after Clearance of Infection. The measurements of antibody levels against *P. yoelii* lysates in sera from mice after clearance of infection were also examined. Large levels of IgM and IgG2a against parasite antigens (Fig. 6 *A*) were observed. We assessed the protective efficacy of the sera after clearance of infection and found a strong degree of protection (Fig. 6 *B*, *right*). Similar results were obtained in mice immunized with GST–MSP1-15 in IFA (data not shown). These results indicated that antibodies induced in the course of infection were protective.

Importance of MSP1-15–specific IFN-γ Production for Protection in Other Kinds of Mice. We next assessed the influence of different genetic backgrounds of mice on level of protection. A/J and C57BL/6 mice were immunized with GST–MSP1-15 in RAS. Before challenge, spleen cells were stimulated with MSP1-15, and the level of IFN-γ production was assessed. The results showed that A/J mice immunized with GST–MSP1-15 produced higher amounts of IFN-γ than C57BL/6 mice when the cells were stimulated with MSP1-15 in vitro (Fig. 7 *A*). Serum was obtained from each mouse before challenge, and the antibody levels against MSP1-15 and their subclasses were examined.

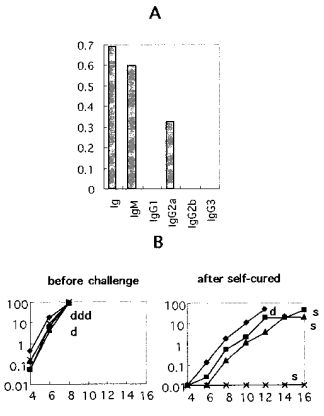

Figure 6. Analysis of sera after challenge. (*A*) Isotype distribution of antibodies against *P. yoelii* lysate. Serum antibodies obtained from surviving mice, which had been preimmunized with rBCGMSP1-15, were assayed for the level of Igs and their distribution into IgM, IgG1, IgG2a, IgG2b, and IgG3 subclasses using anti–mouse subclass-specific antibodies. Sera were diluted to 1:100. Each point represents the average of six surviving animals. (*B*) Passive immunity imparted by transfer of each serum. Sera from C3H/He mice immunized with rBCGMSP1-15 were transferred into groups of four mice before parasite challenge (*left*) and after clearance of infection (*right*). Each line shows the course of parasitemia of an individual mouse. *y-axis*, Percentage of parasitemia. *x-axis*, Days from infection. *d*, Dead mice. *s*, Self-cured mice.

The data show that both strains of mice produced high levels of MSP1-15–specific antibodies. A/J mice produced MSP1-15–specific IgM and IgG2a antibodies, and C57BL/6 mice produced MSP1-15–specific IgM and IgG2b antibodies (Fig. 7 *B*), indicating that GST–MSP1-15 immunization induces the production of IFN-γ and IgG2a in A/J mice but not in C57BL/6 mice. Next, the protective efficacy induced by GST–MSP1-15 immunization in both strains of mice was examined. As shown in Fig. 7 *C*, strong protection was induced only in A/J mice. These data again confirmed that the production of IFN-γ induced by MSP1-15 has a major role in effective protection.

Discussion

The selection of proper antigens and adjuvants is the most important step in developing a successful malaria vaccine. The COOH-terminal fragment of MSP1 has been evaluated as a leading candidate for the malaria vaccine, and BCG has been recognized to have a prominent adjuvant effect. Based on our aim to combine the two, we constructed rBCG which expressed MSP1-15 as a fusion protein with α-k. α-k is a secretory protein of *M. kansasii*; consequently, the fusion protein was expected to be secreted from BCG. The results of Western blotting revealed that MSP1-15 was secreted as a fusion protein, with a similar antigenic conformation as the native COOH-terminal fragment of MSP1-15 with two epidermal growth factor–like domains (Fig. 1 *C*).

Among the proteins of blood-stage *Plasmodium*, MSP1 has been studied the most intensively as a potential target for protective immunity. Two antibody-dependent mechanisms have been reported for protection by immunization with MSP1 against *P. falciparum* cultured in vitro. One is interruption of parasite invasion into erythrocytes by antibodies to the COOH-terminal fragment of MSP1 (35), and the other is inhibition of secondary processing of MSP1 by antibodies (42). Studies supporting antibody-dependent protection induced by MSP1 were reported in the rodent model (50, 51). However, other experiments showed that protection induced by vaccination with MSP1 did not correlate with antibody levels or in vitro growth inhibition assay, using MSP1-immunized sera in rodent (52) and primate models (32, 34, 53). No protective efficacy against *P. falciparum* was observed in the Aotus monkey, though a high titer of MSP1-specific antibody was produced (54). These data suggest that protective mechanisms may vary depending on the immunization protocol and the animals. They also indicate that immunization with MSP1 induced protective effectors other than antibody. High levels of antibody against MSP1-15 before challenge were observed in mice immunized with GST–MSP1-15 in artificial adjuvants (Fig. 5 *C*). However, the sera did not induce protection in vivo (Fig. 6 *B*). Furthermore, rBCGMSP1-15 im-

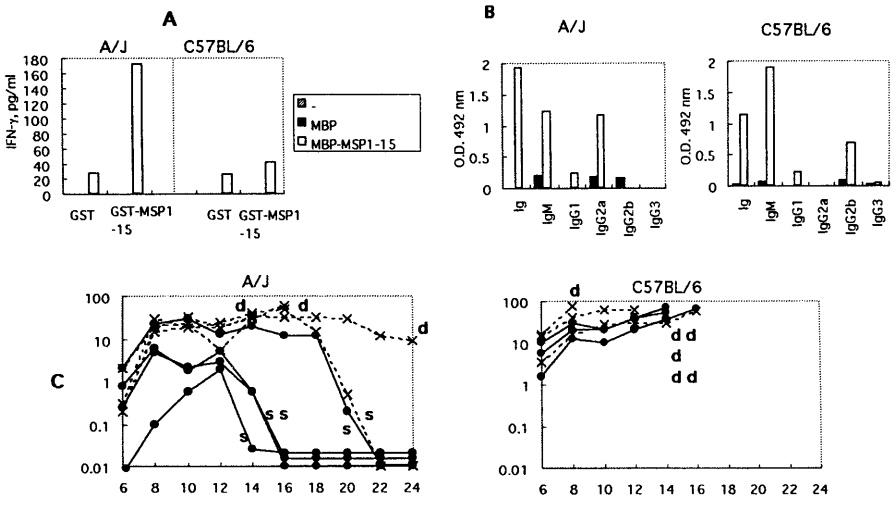

Figure 7. Correlations among cytokine production (*A*), antibody subclass (*B*), and protective efficacy induced by immunization with MSP1-15 (*C*). (*A*) Production of IFN-γ in spleen cells stimulated with control saline (*gray bar*), MBP (*black bar*), and MBP–MSP1-15 (*white bar*). *y-axis*, Amounts of produced cytokines in supernatants. Immunizing samples are indicated below the x-axis. Protocol of IFN-γ measurement is described in Materials and Methods. Assay was performed in duplicate with independent samples; values shown are the averages. (*B*) The antibody levels against MBP–MSP1-15 and their isotype distribution in sera from A/J (*left*) or C57BL/6 (*right*) mice immunized with GST (*black bar*) or GST–MSP1-15 (*white bar*) were analyzed by ELISA. (*C*) Parasitemia curves of *P. yoelii* in A/J (*left*) and C57BL/6 (*right*) mice which had been immunized with GST (X) or GST–MSP1-15 (*filled circle*) in RAS were estimated from Giemsa-stained smears. Each line shows the course of parasitemia of an individual mouse. *y-axis*, Percentage of parasitemia. *x-axis*, Days after infection. *d*, Dead mice. *s*, Self-cured mice.

munization, which was more effective than artificial adjuvants (Fig. 2), did not produce an obvious MSP1-15–specific antibody before *P. yoelii* infection (Fig. 5 *A*). These results indicated that protective efficacy by MSP1-15 immunization did not depend on the MSP1-15–specific antibody, at least at the beginning of infection, in our study.

rBCGMSP1-15 immunization induced more efficient protection in mice than artificial adjuvant systems (Fig. 2). An appreciable amount of IFN-γ was detected when spleen cells from mice immunized with rBCGMSP1-15 were stimulated with MSP1-15 (Fig. 3). Neutralization of IFN-γ at the early stage of *P. yoelii* infection reduced protection. A correlation between protection and production of MSP1-15–specific IFN-γ was observed when A/J and C57BL/6 mice were immunized with GST–MSP1-15 in RAS (Fig. 7). Immunization with rBCGMSP1-15 also protected A/J mice but not C57BL/6 mice, though only parental BCG immunization prevented the death of A/J mice (data not shown). Together, these data indicated that IFN-γ has an important role in the protection induced by MSP1-15 immunization. IFN-γ, which is expected to be produced by several kinds of cells, such as the Th1-type T lymphocyte, $CD8^+$ T lymphocyte, and natural killer cell, mediates cellular immunity by activation of macrophages and enhancement of killing of intracellular organisms (55). IFN-γ also enhances production of NO, which is effective against intracellular parasites (56). It was reported previously that the level of IFN-γ in the sera of monkeys immunized with MSP1 correlated with protection against *P. falciparum* (34). The mechanisms of protection against the blood-stage malaria parasite were studied extensively in the *Plasmodium chabaudi* model using a nonlethal strain (57–60). Those studies revealed that the Th1-type immune response, including IFN-γ and NO, induced protection against *P. chabaudi*. Cellular immunity such as the mononuclear phagocytic system played an important role in protective immunity against intraerythrocytic asexual stages of parasites (61, 62). One of the contributions of MSP1-15–specific IFN-γ for this protection may be to enhance the killing of parasites by activation of the cellular immune response.

In this study, passive transfer experiments showed that sera from self-cured mice after infection were protective (Fig. 6 *D*), and that the major antibody subclass of the protective sera was IgG2a (Fig. 5 *B*). The IgG2a subclass antibody is cytophilic and shows extensive complement-fixing ability. This cytophilic antibody promotes phagocytosis, antibody-dependent cell-mediated cytotoxicity, and antibody-dependent cellular inhibition. It has been implicated in antibody-mediated protective immunity against blood-stage *P. falciparum* in vitro (61). The isotype content of sera from individuals with defined clinical states of resistance or susceptibility to malaria has been investigated in several tropical countries. Those studies also revealed that the cytophilic antibody subclasses predominated in protected subjects (63–65). It has also been reported that the antibody was necessary to eliminate a blood-stage malaria parasite infection (66). Together, these suggest that the cooperation of cytophilic antibody and cell-mediated cytotoxicity is effective for attacking parasites, and that such an immune response might eventually eradicate the parasites. It is believed that IgG2a is the antibody induced by B lymphocytes stimulated with IFN-γ. In this experiment, one of the contributions of MSP1-15 to protection may be to enhance smooth production of protective IgG2a antibodies by IFN-γ stimulation.

In summary, the most important contribution of this study is that we first succeeded in inducing a strong degree of protection against *Plasmodium* infection using rBCG and found that live BCG is the superior adjuvant for a malaria vaccine. The second most important contribution is that IFN-γ plays an important role in the protection induced by MSP1-15 regardless of the adjuvants used to date; it may stimulate the cellular immune response and the production of protective antibodies against the protective antigens of parasites. Thus, we found that both cellular and humoral immune responses were essential for protection against disease. BCG induces strong cellular immunity and has been used all over the world. Therefore, a combination of BCG and parasite antigens may be the best system, and can be safely used in humans. This study is likely to have wide-ranging implications for efforts to develop an effective vaccine against blood-stage *Plasmodium*.

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