Long-Lasting Protective Immune Response to the 19-Kilodalton Carboxy-Terminal Fragment of Plasmodium yoelii Merozoite Surface Protein 1 in Mice

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Received 20 October 2006/Returned for modification 22 December 2006/Accepted 8 February 2007

Merozoite surface protein 1 (MSP1) is the major protein on the surface of the plasmodial merozoite, and its carboxy terminus, the 19-kDa fragment (MSP119), is highly conserved and effective in induction of a protective immune response against malaria parasite infection in mice and monkeys. However, the duration of the immune response has not been elucidated. As such, we immunized BALB/c mice with a standard four-dose injection of recombinant Plasmodium yoelii MSP119 formulated with Montanide ISA51 and CpG oligodeoxynucleotide (ODN) and monitored the MSP119-specific antibody levels for up to 12 months. The antibody titers persisted constantly over the period of time without significant waning, in contrast to the antibody levels induced by immunization with Freund’s adjuvant, where the antibody levels gradually declined to significantly lower levels 12 months after immunization. Investigation of immunoglobulin G (IgG) subclass longevity revealed that only the IgG1 antibody level (Th2 type-driven response) decreased significantly by 6 months, while the IgG2a antibody level (Th1 type-driven response) did not change over the 12 months after immunization. After challenge infection, all immunized mice survived with negligibly patent parasitemia. These findings suggest that protective immune responses to MSP119 following immunization using oil-based Montanide ISA51 and CpG ODN as an adjuvant are very long-lasting and encourage clinical trials for malaria vaccine development.

Malaria is a major infectious disease that results in severe morbidity and mortality. Recently, it has been estimated that 2.2 billion people worldwide are exposed to Plasmodium falciparum and 515 (ranging from 300 to 660) million individuals had clinical episodes of malaria in 2002 (24). Many factors are involved in this burden of malaria, such as the appearance of drug-resistant strains of Plasmodium, both P. falciparum and P. vivax, insecticide-resistant Anopheles mosquito vectors, and the lack of an effective malaria vaccine (8). Many malaria vaccine candidates have been developed, and some of them are being tested in ongoing clinical trials (6, 7).

Merozoite surface protein 1 (MSP1) is a leading malaria vaccine candidate. It is produced during schizogony and merozoite maturation. MSP1 is composed of many fragments, and only its small 19-kDa fragment at the carboxyl terminus (MSP119) is carried into newly infected erythrocytes (2). MSP119 is highly conserved and is composed of two epidermal growth factor-like domains which contain protective epitopes (5, 17). In previous studies, it was shown that immunization with recombinant MSP119 of Plasmodium falciparum or P. yoelii protects monkeys or mice, respectively, against infection (5, 10, 15, 17). Our studies have also shown that protection is correlated with high levels of MSP119-specific antibodies at the time prior to challenge infection, but not with effector T cells or other accessory factors associated with cell-mediated immunity (10, 11). Passive transfer of MSP119-immune serum has demonstrated that while an active immune response postinfection is necessary for protection against lethal malaria (12), its specificity for MSP119 is not required for protection (29).

CpG oligodeoxynucleotides (ODNs) have extensive activity to activate the innate and adaptive immune responses (14) via binding to Toll-like receptor 9 (9). Activation of dendritic cells by CpG ODN induces cell maturation and production of proinflammatory cytokines, such as interleukin 1 (IL-1), IL-6, tumor necrosis factor alpha, and type I interferon, as well as Th1-promoting cytokine IL-12 (1, 25). CpG ODNs have been found to be useful as adjuvants for peptide/protein vaccines against various pathogens, including malaria parasite antigens (13, 16, 19, 26). The results of our previous studies have demonstrated that CpG ODN in combination with Montanide ISA51 or ISA720 strongly promotes MSP119 in induction of specific antibody response and protection against a lethal malaria infection in mice (13). However, the longevity of the antibody response to MSP119 has not been studied. In this study we investigate how long the MSP119-specific antibody response lasts following immunization with recombinant P. yoelii MSP119 formulated with CpG ODN in Montanide ISA51 and the kinetics of the antibody isotype responses, as well as the degree of protection.

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7 Published ahead of print on 21 February 2007.
MATERIALS AND METHODS

Mice and parasites. Female BALB/c mice, 6 to 8 weeks of age at the start of the experiments, were purchased from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakhon Prathom, Thailand. *Plasmodium yoelii* YM, a lethal murine malaria parasite, was maintained in our laboratory and used for challenge infection.

Recombinant MSP1<sub>19</sub> protein. Recombinant MSP1<sub>19</sub> protein of *P. yoelii* YM was produced as *Saccharomyces cerevisiae*-expressed FLAG fusion protein (FLAG-MSP1<sub>19</sub>) according to the instructions of the manufacturer (Eastman Kodak, Scientific Imaging Systems). The recombinant protein was purified using an anti-FLAG M1 antibody gel column (Sigma), and its purity was demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by formation of a single band (13).

Adjuvants. CpG ODN 1826 (TCCATGACGTTCCTGACGTT; the two C6 motifs are underlined) used in this study was kindly provided by A. M. Krieg, Coley Pharmaceutical Group. Montanide ISA51 was a kind gift from SEPPIC, France. Complete Freund’s adjuvant (CFA) and incomplete Freund’s adjuvants (IFA) were obtained from Sigma.

Immunization protocol. Mice were immunized subcutaneously with an emulsion of the mixture of one part of phosphate-buffered saline (PBS) or 20 μg of recombinant MSP1<sub>19</sub> plus 50 μg CpG ODN 1826 and one part of Montanide ISA51 or of one part of PBS or the antigen and one part of CFA. On days 21, 48, and 56, mice were boosted with the same amount of antigen plus CpG ODN in Montanide ISA51 or with the antigen plus IFA via subcutaneous, intraperitoneal (i.p.), and i.p. injections, respectively (10, 13).

Antibody assay. Sera were collected 2 weeks after the last immunization and then every month for the assessment of MSP1<sub>19</sub>-specific immunoglobulin G (IgG) antibody and antibody subclasses by enzyme-linked immunosorbent assay (ELISA) as described previously (13). Briefly, MaxiSorb immunoplates (Nunc, Denmark) were coated with 100 μl of 0.5 μg/ml MSP1<sub>19</sub> in coating buffer overnight at 4°C. After three washes with 0.05% Tween 20 in PBS, wells were blocked by the addition of 200 μl of PBS containing 1% bovine serum albumin at 37°C for 1 h. Supernatants were discarded, and 100-μl amounts of twofold serial dilutions of serum were added to the wells. After incubation for 1 h, the wells were washed, and then 100 μl of horseradish peroxidase-conjugated goat anti-mouse IgG (Zymed Laboratories, Inc.) diluted 1/3,000 was added to each well. For antibody subclass determination, after incubation with sera and washing the well, 100 μl of horseradish peroxidase-conjugated goat anti-mouse IgG1 or IgG2a (Zymed Laboratories, Inc.) diluted 1/1,000 was added to each well. After incubation for 1 h, the wells were washed and incubated for 30 min, and then 100 μl of 1 N H<sub>2</sub>SO<sub>4</sub> was added to stop the reaction. The plate was read for optical density (OD) at 490 nm using an ELISA reader. The antibody titers were judged as the highest dilution of serum for which the OD was equal to or greater than the mean OD of healthy control sera.

Challenge infection. Mice were challenged intravenously with 1 × 10<sup>6</sup> live *P. yoelii* YM-parasitized red blood cells (YM-pRBC). Parasitemia was monitored daily by microscopic examination of Dip-Quick-stained blood films, counting at least 10,000 RBC before declaring a slide to be negative.

Statistical analysis. The significance of differences between values was determined by Student’s t test of Sigma Plot window version 9.0 (SPSS).

RESULTS

Immunization with recombinant MSP1<sub>19</sub> plus CpG ODN in Montanide ISA51 induces strong antibody responses. We have shown previously that immunization with MSP1<sub>19</sub> formulated with CpG ODN 1826 in Montanide ISA51 (CpG ODN/ISA) induces a very high antibody response and confers complete protection against *P. yoelii* YM infection (13). In the current study we used this immunization regimen to investigate the duration of the protective immune response. For comparison, we used CFA or IFA as an adjuvant. Mice were immunized with four injections of MSP1<sub>19</sub> mixed with CpG ODN/ISA at days 1, 21, 42, and 56. Fourteen days after the last immunization, sera were collected and assayed for MSP1<sub>19</sub>-specific IgG antibody by ELISA. Results showed that IgG antibody responses in mice immunized with MSP1<sub>19</sub> and CpG ODN/ISA were higher than those in mice immunized with MSP1<sub>19</sub> in CFA/IFA as demonstrated by OD of sera at the dilution of 1/1,000,000 (mean OD ± standard error [SE], 1.260 ± 0.169 versus 0.351 ± 0.044; *P* < 0.001) (Fig. 1A) and by antibody titers (geometric mean ± SE, 7.204 ± 0.199 versus 6.401 ± 0.072; *P* < 0.01) (Fig. 1B). Furthermore, the IgG1 and IgG2a antibody subclass responses were higher following immunization with MSP1<sub>19</sub> plus CpG ODN/ISA compared to the use of CFA/IFA as an adjuvant (geometric mean ± SE of IgG1 antibody titers, 7.330 ± 0.283 versus 6.693 ± 0.177; *P* < 0.01) (Fig. 1C). To compare the potency of CpG ODN/ISA and CFA/IFA in initiating IgG1 and IgG2a antibody production, we converted the log titers to the original antibody titers, i.e., log titer of 7.330 was converted to 21,374,698 and 6.693 to 4,926,517 for IgG1 antibodies and from 6.305 to 2,016,043 and 4.407 to 25,541 for IgG2a antibodies and then determined the ratio for each subclass. We found that for MSP1<sub>19</sub>/CpG ODN/ISA and MSP1<sub>19</sub>/CFA/IFA immunizations, the ratio of IgG1 antibody titers was 4.3 (21,374,698/4,926,517) whereas that of IgG2a antibody titers was 79.0 (2,016,043/25,541), suggesting that CpG ODN/ISA51 induces a very high antibody response and confers complete protection against *P. yoelii* YM infection (13). In the current study we used this immunization regimen to investigate the duration of the protective immune response. For comparison, we used CFA or IFA as an adjuvant. Mice were immunized with four injections of MSP1<sub>19</sub> mixed with CpG ODN/ISA at days 1, 21, 42, and 56. Fourteen days after the last immunization, sera were collected and assayed for MSP1<sub>19</sub>-specific IgG antibody by ELISA. Results showed that IgG antibody responses in mice immunized with MSP1<sub>19</sub> and CpG ODN/ISA were higher than those in mice immunized with MSP1<sub>19</sub> in CFA/IFA as demonstrated by OD of sera at the dilution of 1/1,000,000 (mean OD ± standard error [SE], 1.260 ± 0.169 versus 0.351 ± 0.044; *P* < 0.001) (Fig. 1A) and by antibody titers (geometric mean ± SE, 7.204 ± 0.199 versus 6.401 ± 0.072; *P* < 0.01) (Fig. 1B). Furthermore, the IgG1 and IgG2a antibody subclass responses were higher following immunization with MSP1<sub>19</sub> plus CpG ODN/ISA compared to the use of CFA/IFA as an adjuvant (geometric mean ± SE of IgG1 antibody titers, 7.330 ± 0.283 versus 6.693 ± 0.177; *P* < 0.01) (Fig. 1C). To compare the potency of CpG ODN/ISA and CFA/IFA in initiating IgG1 and IgG2a antibody production, we converted the log titers to the original antibody titers, i.e., log titer of 7.330 was converted to 21,374,698 and 6.693 to 4,926,517 for IgG1 antibodies and from 6.305 to 2,016,043 and 4.407 to 25,541 for IgG2a antibodies and then determined the ratio for each subclass. We found that for MSP1<sub>19</sub>/CpG ODN/ISA and MSP1<sub>19</sub>/CFA/IFA immunizations, the ratio of IgG1 antibody titers was 4.3 (21,374,698/4,926,517) whereas that of IgG2a antibody titers was 79.0 (2,016,043/25,541), suggesting that CpG ODN/ISA51
is highly potent in induction of IgG2a antibody (or Th1 type) response. Control mice immunized with PBS instead of MSP119 did not induce MSP119-specific antibody responses.

**Immunization with recombinant MSP119 plus CpG ODN/ISA induces long-lasting antibody responses.** To determine how long the MSP119-specific antibody lasts, after the last immunization, sera were collected and antibody levels measured every month for 12 months. We found that the MSP119-specific antibody titers in mice immunized with MSP119 plus CpG ODN/ISA persisted without any significant change over 12 months (geometric mean ± SE, 6.687 ± 0.261 versus 6.904 ± 0.147 at 1 and 12 months, respectively, after the last immunization) (Fig. 2). In contrast, the antibody titers in mice immunized with MSP119 in CFA/IFA gradually decreased over the 12 months after the last immunization, and compared to the antibody levels in the first month after immunization, the antibody levels at 12 months were significantly lower (geometric mean ± SE, 0.465 ± 0.118 versus 0.577 ± 0.100 at 1 and 12 months, respectively; P < 0.0001) (Fig. 2).

We also investigated the duration of MSP119-specific IgG1 and IgG2a antibody subclass responses for 12 months after immunization. Interestingly, the IgG1 antibody responses in both groups of mice immunized with MSP119 plus CpG ODN/ISA and MSP119 in CFA/IFA decreased continuously, and the levels at 6 and 12 months were significantly lower than the levels at 1 month after immunization (mean OD ± SE, 2.660 ± 0.131 and 1.634 ± 0.152 versus 1.024 ± 0.158 [P < 0.001 and P < 0.0001, respectively, when using CpG ODN/ISA as adjuvant]; values of 1.552 ± 0.084 and 0.870 ± 0.116 versus 0.501 ± 0.212 [P < 0.01 and P < 0.01 when using CFA/IFA as adjuvant at month 1, 6, and 12 postimmunization, respectively] (Fig. 3A). In contrast, the IgG2a antibody responses of both immunizations were stable over 12 months postimmunization, and the antibody levels were not significantly different compared at 6 and 12 months to the level the first month postimmunization (mean OD ± SE, 1.317 ± 0.066, 1.249 ± 0.049, and 1.213 ± 0.057, when using CpG ODN/ISA as adjuvant and values of 0.687 ± 0.060, 0.689 ± 0.057, and 0.684 ± 0.039 when using CFA/IFA as adjuvant at month 1, 6, and 12 postimmunization, respectively) (Fig. 3B). However, the titers of antibody following immunization with CpG ODN/ISA were always higher than those after immunization with CFA/IFA.

**Long-lasting protective MSP19-specific immune response against P. yoelii challenge infection.** Having shown above that immunization with MSP119 plus ODN/ISA or CFA/IFA induced and maintained the vaccine-specific antibody for at least 12 months, we then examined whether the immunity was protective by the end of this period. Mice were challenged with 1 × 10⁷ P. yoelii YM-pRBC intravenously, and parasitemia was monitored. It was found that all mice immunized with MSP119 plus CpG ODN/ISA survived infection with no detectable parasitemia (Fig. 4D). Mice immunized with MSP119 plus CFA/IFA were less protected; one mouse was completely protected, two mice experienced patent parasitemia (<1%) before they recovered from the infection, and one mouse showed delayed infection for 7 days and died with high parasitemia by day 18 postinfection (Fig. 4C). All control mice immunized with PBS plus CpG ODN/ISA or CFA/IFA died within 8 days with high parasitemia, except one mouse immunized with PBS in CFA/IFA that recovered after being infected in the same manner as the other control mice (Fig. 4A and B).

**DISCUSSION**

Vaccines need to be highly efficacious in protection against a pathogen and induce long-lasting protective immune response. Our previous studies have shown that immunization of mice with MSP119 formulated with Freund's adjuvants induces high titers of antibody, which completely protects against a lethal malaria infection (10). Recently, we have demonstrated that the formulation of MSP119 with CpG ODN 1826 and Montanide ISA51 or ISA720 induces a more effective antibody response. Control mice immunized with PBS instead of MSP119 did not induce MSP119-specific antibody responses.

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response and protection against lethal malaria infection compared to the formulation of MSP1,9 with Montanide ISA51 or ISA720 alone, or with Freund’s adjuvant, suggesting that CpG ODN plays a role in immunological enhancement (13). Here in this study, we then examined the longevity of MSP1,9-specific immunity induced by immunization with MSP1,9 formulated with CpG ODN 1826 in Montanide ISA51. We also used the vaccine formulation with CFA/IFA for comparison. The results showed that total IgG antibody specific for MSP1,9 and protection lasted over 12 months after immunization and that the Th1-dependent IgG2a antibody response persisted stably, while the Th2-dependent IgG1 antibody response declined over the period of time in either vaccine formulation.

Freund’s adjuvant is an oil-based immunostimulatory agent, effectively used in animal immunization to initiate a protective immune response. The use of this adjuvant in a formulation with MSP1,9 induces high MSP1,9-specific antibody response and confers complete protection against P. yoelii YM infection (10). The use of Freund’s adjuvant is not allowed for human vaccination because of its toxicity. Montanide ISA51 is an oil-based adjuvant of higher purity than Freund’s adjuvant and has been demonstrated to be safe for use in humans (20). In a mouse system, MSP1,9 formulated with Montanide ISA51 induces protection against P. yoelii infection even though some mice experienced some patent parasitemia, but in the presence of CpG ODN 1826, complete protection is obtained. This enhancement of protection is correlated with increases in the IgG1 and IgG2a subclass responses (13). The increases of both antibody subclasses are also greater than those induced by using Freund’s adjuvant, which we again have confirmed in this study (Fig. 1). Moreover, the increase of IgG2a antibody titers was much greater than that of IgG1 antibody levels, leading to the suggestion that the formulation with CpG ODN in Montanide ISA51 preferentially stimulates the Th1-dependent antibody response.

In this study, MSP1,9-specific IgG antibody titers of four mice at 12 months following the last immunization with MSP1,9 plus CFA/IFA were 421,599, 700,489, 621,767, and 664,660, and after challenge infection, the mice had prepatent periods of 11, 8, 10, and 6 days with peak parasitemia of 0.1, 1.17, 0.44, and >47% (death), suggesting that the prechallenge antibody titers are critical for the protection outcome. Those antibody titers were decreased about fivefold compared to the antibody titers at first month postimmunization. These results are consistent with the results of our previous studies, which demonstrated that mice immunized with MSP1,9 plus CFA/IFA with prechallenge MSP1,9-specific antibody titers of >6,400,000 were completely protected from challenge infection (10), and that as demonstrated by intranasal immunization, the antibody titers of <640,000 could not confer protection against infection and mice died with high parasitemia, whereas mice that had antibody titers from 640,000 to 2,256,000 experienced little patent parasitemia (<1%) and survived infection (11).

In both CpG ODN/ISA51 and CFA/IFA groups, MSP1,9-specific IgG1 subclass levels continuously decreased, whereas the IgG2a subclass stably persisted over 12 months postimmunization (Fig. 3). This led to a question why the decrease of IgG1 antibody did not affect the total IgG antibody level, particularly in CpG ODN/ISA51 group (Fig. 2). This may be explained by the following reasons. First, both IgG1 and IgG2a antibody titers produced by the CpG ODN/ISA51 group were much higher than the titers produced by the CFA/IFA group; IgG1 titers were 21,374,698 and 4,926,517, and IgG2a titers were 2,016,043 and 25,541, respectively, making the IgG1 and IgG2a ratios of 11 (21,374,698/2,016,043) and 193 (4,926,517/
The level waned significantly within 22 weeks. Protective antibody level induced by four-dose immunization, or CFA was able to yield an antibody level comparable to the group. First, our previous study demonstrated that immunization of individuals in areas where malaria is endemic would aid in immunity against malaria infection (23). In this paper, we have further shown that those immunizations can give rise to long-term protection, which persists for at least 12 months, suggesting that anamnestic immunization is critical for long-lasting immune responses. Fewer doses of immunization which yielded lower antibody levels and less protection (10) would be less effective in maintenance of the antibody response. Our recent unpublished data showed that a single-dose immunization (one injection) with MSP119 in CpG ODN/ISA51 or CFA was able to yield an antibody level comparable to the protective antibody level induced by four-dose immunization, but the level waned significantly within 22 weeks.

Long-term response of MSP119-specific antibody may reflect the generation and maintenance of long-lived plasma cells which survive for years (18, 22). However, it has been recently demonstrated that mouse memory B cells and long-lived plasma cells specific for P. yoelii MSP119, were deleted by apoptosis during malaria infection (30). Our data provide new insight into the role of MSP119-specific antibody response by showing that MSP119-specific IgG2a antibody persists constantly longer than IgG1 antibody (Fig. 3A and B) and suggest that the memory of MSP119-specific IgG2a antibody may last longer than the IgG1 memory.

Despite the success of immunization in mice, some researchers have raised the arguments that multiple injections may be difficult for use in humans, particularly in younger children who are many of the targets, that the i.p. route is not suitable for human injection and an alternative route is needed, and that another CpG ODN(s) which is the most potent adjuvant is used separately in human vaccine trials are needed for novel strategies. Trends Parasitol. 21:29–34.

In summary, we have demonstrated that MSP119 immunization formulated with CpG ODN and Montanide ISA51 induces long-term antibody responses and confers complete protection against blood-stage malaria parasite infection. Second, MSP119-specific IgG2a antibody stably persists longer than the IgG1 antibody. Recently, Montanide ISA51 and CPG 7909, a B-class CpG ODN, used separately in human vaccine trials are well-tolerated and enhance vaccine immunogenicity (4, 20). The combination of Montanide ISA51 and CpG ODN adjuvants should be tested in a clinical trial.

ACKNOWLEDGMENTS

This work was supported by UNDP/World Bank/World Health Organization Specific Programme for Research and Training in Tropical Diseases and China Medical Board, Faculty of Public Health, Mahidol University, Thailand.

We thank A. M. Krieg for the gift of CpG ODN 1826 and SEPPIC, France, for Montanide ISA51. We are indebted to Michael F. Good and Michelle Wykes for critically reading the manuscript.

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