Long Lasting Protective Immune Response to 19 kDa Carboxy-Terminal Fragment of *Plasmodium yoelii* Merozoite Surface Protein 1 (MSP1\textsubscript{19}) in Mice

Pimmada Jeamwattanalert,\textsuperscript{1} Yuvadee Mahakunkijcharoen,\textsuperscript{2} Leera Kittigul,\textsuperscript{1} Pakpimol Mahannop,\textsuperscript{3} Sathit Pichyangkul,\textsuperscript{4} and Chakrit Hirunpetcharat\textsuperscript{1*}

Department of Microbiology, Faculty of Public Health, Mahidol University,\textsuperscript{1} Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University,\textsuperscript{2} Department of Parasitology, Faculty of Public Health, Mahidol University,\textsuperscript{3} Department of Immunology and Medicine, US Armed Forces Research Institute of Medical Science, Bangkok, Thailand\textsuperscript{4}

\* Corresponding author. Mailing address: Department of Microbiology, Faculty of Public Health, Mahidol University, 420/1 Rajvithi Road, Bangkok 10400, Thailand. Phone: (662) 3548528 ext. 104. Fax: (662) 3548538. E-mail: phchr@mahidol.ac.th.

Running title: Longevity of immunity to MSP1

Keywords: immunity, malaria, MSP1, *Plasmodium yoelii*
Abstract

MSP1 is the major protein on the surface of plasmodial merozoite and its carboxy-
terminus, the 19kDa-fragment (MSP1\textsubscript{19}), 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 \textit{P. yoelii} MSP1\textsubscript{19} formulated with Montanide ISA51 and CpG oligodeoxynucleotide (ODN) and monitored the MSP1\textsubscript{19}-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 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 post immunization but that the boosting effect was seen in the IgG1 but not in IgG2a antibody responses. After challenge infection, all immunized mice survived with negligibly patent parasitemia. These findings suggest that protective immune responses to MSP1\textsubscript{19} following immunization using oil-based Montanide ISA51 and CpG ODN as adjuvant are very long-lasting and encourage clinical trials for malaria vaccine development.
Introduction

Malaria is a major infectious disease which results in severe morbidity and mortality. Recently, it has been estimated that 2.2 billion people worldwide are exposed to *P. 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 of *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 ongoing in clinical trials (6,7).

Merozoite surface protein-1 (MSP1) is a leading malaria vaccine candidate. It is produced during schizogony and merozoite maturation, composed of many fragments, and only its small fragment of 19kDa at the carboxyl terminus is carried into newly uninfected erythrocytes (1). This fragment, namely MSP1$_{19}$, is highly conserved and is composed of two epidermal growth factor (EGF)-like domains which contain protective epitopes (5,17). In previous study, it was shown that immunization with recombinant MSP1$_{19}$ 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 MSP1$_{19}$-specific antibodies at the time prior to challenge infection, but not with effector T cells nor other accessory factors associated with cell-mediated immunity (10,11). Passive transfer of MSP1$_{19}$-immune serum has demonstrated that while an active immune response post infection is necessary for protection against lethal malaria (12), its specificity for MSP1$_{19}$ is not required for protection (29).

CpG ODNs have extensive ability to activate the innate and adaptive immune responses (14), via binding to Toll-like receptor (TLR)-9 (9). Activation of dendritic cells (DC) by CpG
ODN induces cell maturation and production of proinflammatory cytokines such as IL-1, IL-6, TNF-α and type I IFN, as well as Th1-promoting cytokine IL-12 (3,25). 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 MSP1<sub>19</sub> in induction of specific antibody response and protection against a lethal malaria infection in mice (13). However, the longevity of antibody response to MSP1<sub>19</sub> has not been studied. Here, in this study we investigate how long the MSP1<sub>19</sub>-specific antibody response lasts following immunization with recombinant <i>P. yoelii</i> MSP1<sub>19</sub> formulated with CpG ODN in Montanide ISA51, the kinetics of the antibody isotype responses, as well as protection.

**Materials and Methods**

**Mice and parasites.** Female BALB/c mice, 6–8 weeks of age at the start of experiments, were purchased from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakhon Prathom, Thailand. <i>Plasmodium yoelii</i> 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 <i>P. yoelii</i> YM was produced as *Saccharomyces cerevisiae*-expressed FLAG-fusion protein (FLAG-MSP1<sub>19</sub>) according to the instruction of the manufacturer (Eastman Kodak, Scientific Imaging Systems). The recombinant protein was purified using an anti-FLAG M1 antibody gel column (Sigma) and the purity was demonstrated by SDS-PAGE to be a single band (13).

**Adjuvants.** CpG ODN #1826 (TCCATGACG<sub>T</sub>TTCCTGACG<sub>T</sub>) used in this study was kindly provided by AM. Krieg, Coley Pharmaceutical Group, USA. 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 (s.c.) with an emulsion of the mixture of one part of PBS or 20µg of recombinant MSP1\textsubscript{19} plus 50µg CpG ODN 1826 and one part of Montanide ISA51, or 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 s.c., intraperitoneal (i.p.), and i.p. injections, respectively (10,13).

**Antibody assay.** Sera were collected two weeks after the last immunization and then every month for the assessment of MSP1\textsubscript{19}-specific IgG antibody and antibody subclasses by enzyme-linked immunosorbent assay (ELISA) as described previously (13). Briefly, MaxiSorb immunoplates (Nunc, Denmark) were coated with 100µl of 0.5µg/ml MSP1\textsubscript{19} in coating buffer overnight at 4°C. After three washes with 0.05% Tween 20 in PBS (PBST), wells were blocked with 200µl of 1% BSA/PBS at 37°C for 1 h. Supernatants were discarded, and 100µl of two-fold serial dilutions of serum was added. After incubation for 1 h, wells were washed and then 100µl of 1/3,000 HRP-conjugated goat anti-mouse IgG (Zymed, Laboratories Inc, USA) was added. For antibody subclass determination, after incubation with sera and being washed 100µl of 1/1,000 HRP-conjugated anti-mouse IgG1 or IgG2a (Zymed, Laboratories Inc, USA) were added into wells. After incubation for 1 h, wells were washed and 100µl of o-phenylenediamine dihydrochloride (OPD; Sigma) substrate solution was added. The plate was incubated at room temperature for 30 min and then added with 100µl of 1N H\textsubscript{2}SO\textsubscript{4} to stop reaction. The plate was read for optical density (O.D.) at 492 nm using an ELISA reader. The antibody titers were
judged as the highest dilution of serum for which the O.D. was equal to or greater than the mean O.D. of normal control sera.

**Challenge infection.** Mice were challenged intravenously with $1 \times 10^4$ live *P. yoelii* YM-parasitized red blood cells (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$_{19}$ plus CpG ODN in Montanide ISA51 induces strong antibody response.** We have shown previously that immunization with MSP1$_{19}$ 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 protective immune response. For comparison we used CFA/IFA as an adjuvant. Mice were immunized with four injections of MSP1$_{19}$ 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$_{19}$-specific IgG antibody by ELISA. Results showed that IgG antibody responses in mice immunized with MSP1$_{19}$ and CpG ODN/ISA were higher than those in mice immunized with MSP1$_{19}$ in CFA/IFA as demonstrated by O.D. of sera at the dilution of 1/1,000,000 (the mean O.D. ± S.E.; 1.260 ± 0.169 vs 0.351 ± 0.044, *p*<0.001 [Fig. 1A]) and by antibody titers (geometric mean ± S.E.; 7.204 ± 0.199 vs 6.401 ± 0.072, *p*<0.01 [Fig.1B]). Furthermore, the IgG1 and IgG2a
antibody subclass responses were higher following immunization with MSP1\textsubscript{19} plus CpG ODN/ISA, compared to the use of CFA/IFA as an adjuvant (geometric mean $\pm$ S.E. of IgG1 antibody titers; 7.330 $\pm$ 0.283 vs 6.693 $\pm$ 0.177, p<0.01; geometric mean $\pm$ S.E. of IgG2a antibody titers; 6.305 $\pm$ 0.748 vs 4.407 $\pm$ 0.427, p<0.01) (Fig. 1C). To compare the potency of CpG ODN/ISA and CFA/IFA in initiating IgG1 and IgG2a antibody production, we took anti-log of geometric mean titers to be 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 of each subclass. We found that between MSP1\textsubscript{19}/CpG ODN/ISA and MSP1\textsubscript{19}/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/ISA\textsubscript{51} is highly potent in induction of IgG2a antibody (or Th1 type) response. Control mice immunized with PBS instead of MSP1\textsubscript{19} did not induce MSP1\textsubscript{19}-specific antibody responses.

**Immunization with recombinant MSP1\textsubscript{19} plus CpG ODN/ISA induces long-lasting antibody responses.** To determine how long the MSP1\textsubscript{19}-specific antibody lasts, after the last immunization, sera were collected and antibody levels measured every month for 12 months. We found that the MSP1\textsubscript{19}-specific antibody titers in mice immunized with MSP1\textsubscript{19} plus CpG ODN/ISA persisted without any significant change over 12 months (geometric mean $\pm$ S.E.; 6.687$\pm$0.261 vs 6.904$\pm$0.147, at 1 and 12 months, respectively, after the last immunization) (Fig. 2). In contrast, the antibody titers in mice immunized with MSP1\textsubscript{19} in CFA/IFA gradually decreased over the 12 months after the last immunization and as compared to the first month.
after immunization the antibody levels at 12 months were significantly lower (geometric mean ± S.E.; 6.465 ± 0.118 vs 5.772 ± 0.100, at 1 and 12 months, respectively, \( p < 0.0001 \)) (Fig. 2).

We also investigated the duration of MSP\(_{19}\)-specific IgG1 and IgG2a antibody subclass responses for 12 months after immunization. Interestingly, the IgG1 antibody responses in both groups of mice immunized with MSP\(_{19}\) plus CpG ODN/ISA and MSP\(_{19}\) 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 O.D. ± S.E.; 2.660 ± 0.131, 1.634 ± 0.152, and 1.024 ± 0.158, \( p < 0.001 \) and \( p < 0.0001 \) when using CpG ODN/ISA as adjuvant; and 1.552 ± 0.084, 0.870 ± 0.116, and 0.501 ± 0.212, \( p < 0.01 \) and \( p < 0.01 \) when using CFA/IFA as adjuvant at month 1, 6, and 12 post immunization, respectively) (Fig. 3A). In contrast, the IgG2a antibody responses of both immunizations were stable over 12 months post immunization and the antibody levels were not significantly different when compared at 6 and 12 months to the first month post immunization (mean O.D. ± S.E.; 1.317 ± 0.066, 1.249 ± 0.049, and 1.213 ± 0.057, when using CpG ODN/ISA as adjuvant; and 0.687 ± 0.060, 0.689 ± 0.037, and 0.684 ± 0.039, when using CFA/IFA as adjuvant at month 1, 6, and 12 post immunization, respectively) (Fig. 3B). However, the titers of antibody following immunization with CpG ODN/ISA was always higher than CFA/IFA.

**Long lasting protective MSP\(_{19}\)-specific immune response against *P. yoelii* challenge infection.** Having shown above that immunization with MSP\(_{19}\) 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 \( 1 \times 10^4 \) *P. yoelii*YM-pRBC i.v. and parasitemia was monitored. It was found that all mice
immunized with MSP19 plus CpG ODN/ISA survived infection with no detectable parasitemia (Fig. 4D). Mice immunized with MSP19 plus CFA/IFA were less protective; one mouse was completely protected, two mice experienced patent parasitemia <1% before they recovered infection, and one mouse showed delayed infection for 7 days and died with high parasitemia by day 18 post infection (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 recovered after getting infection in the same manner as other controls (Fig. 4A, B).

**Discussion**

Vaccines need to be highly efficacious in protection against a pathogen as well as induce long lasting protective immune response. Our previous studies have shown that immunization of mice with MSP19 formulated with Freund’s adjuvants induces high titers of antibody which completely protects against a lethal malaria infection (10). Recently, we have demonstrated that formulation of MSP19 with CpG ODN#1826 and Montanide ISA51 or ISA720 induces a more effective antibody response and protection against lethal malaria infection, compared to the formulation of MSP19 with Montanide ISA51 or ISA720 alone, or with Freund’s adjuvant, suggesting the role of CpG ODN on immunological enhancement (13). Here in this study, we then examined the longevity of MSP19-specific immunity induced by immunization with MSP19 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 MSP19 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 formulations.
Freund’s adjuvant is an oil-based immunostimulatory agent, effectively used in animal immunization to initiate a protective immune response. Using this adjuvant to formulate with MSP1\textsubscript{19} induces high MSP1\textsubscript{19}-specific antibody response and confers complete protection against \textit{P. yoelii} YM infection (10). The use of Freund’s adjuvant is not allowed for human vaccination because of its toxicity. Montanide ISA51 is a more purified oil-based adjuvant than the Freund’s adjuvant and has been demonstrated to be safe for use in humans (20). In a mouse system, MSP1\textsubscript{19} formulated with Montanide ISA51 induces protection against \textit{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 the increase of 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 these findings in this study (Fig. 1). Moreover, the increase of IgG2a antibody titers was much greater than that of IgG1 antibody levels, leading to a suggestion that the formulation with CpG ODN in Montanide ISA51 prefers to stimulate Th1-dependent antibody response.

In this study, MSP1\textsubscript{19}-specific IgG antibody titers of four mice at 12 months following the last immunization with MSP1\textsubscript{19} + CFA/IFA were 421,599, 700,489, 621,767, and 664,660 and after challenge infection the mice had pre-patent periods of 11, 8, 10 and 6 days with peak parasitemia of 0.1, 1.17, 0.44, and >47% (death), suggesting that the pre-challenge antibody titers are critical for the protection outcome. Those antibody titers were about five-fold decreased, compared to the antibody titers at first month post immunization. These results are consistent with our previous studies which demonstrated that mice immunized with MSP1\textsubscript{19}+CFA/IFA with pre-challenge MSP1\textsubscript{19}-specific antibody titers >6,400,000 were completely protected from challenge infection (10), and that as demonstrated by intranasal
immunization the antibody titers <640,000 could not confer protection against infection and mice
died with high parasitemia whereas mice that had antibody titer 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\textsubscript{19}-specific IgG1 subclass levels
continuously decreased whereas the IgG2a subclass stably persisted over 12 months post
immunization (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 were much
higher produced in CpG ODN/ISA51 group than in 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 (4,926,517/25,541), respectively.
The IgG2a antibody titer in CpG ODN/ISA51 group was about 80 folds higher than in CFA/IFA
group. Second, the other IgG subclasses such as IgG2b may also be produced prominently as
had been demonstrated by Kumar et al (2004). Therefore, the loss of some IgG1 antibody but
with the persistent of high levels of IgG2a and IgG2b antibodies (Th1 type response) may not
significantly affect to total IgG antibody titer in CpG ODN/ISA51 group. Third, we think that
analysis of antibody level by measurement of O.D. at one dilution of serum would be more
sensitive than the assessment of antibody titer.

Immunization with four doses of MSP1\textsubscript{19} either adjuvanted with CFA/IFA or CpG
ODN/ISA has been shown to give complete protection against \textit{P. yoelii} infection (10,13). 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 MSP\textsubscript{19} in CpG ODN/ISA or CFA was able to yield 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 MSP\textsubscript{19}-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-lives plasma cells specific for \textit{P. yoelii} MSP\textsubscript{19} were deleted by apoptosis during malaria infection (30). Our data provide a new insight into the role of MSP\textsubscript{19}-specific antibody response that MSP\textsubscript{19}-IgG2a antibody persists constantly longer than IgG1 antibody (Fig. 2B, C) and suggest that the memory of MSP\textsubscript{19}-specific IgG2a antibody may last longer than the IgG1 memory.

Despite the success of immunization in mice, some arguments are raised that multiple injections may be difficult for use in humans, particularly in younger children who are most targets, that i.p. route is not suitable for human injection and alternative route is needed, and that other CpG ODN(s) which are mostly potent adjuvant in humans would be sought to achieve protective immunity. Therefore, the research on minimizing doses (to one or two injections) and natural boosting by malaria infection of the vaccinated individuals in malaria endemic areas would be encouraged for malaria vaccine development.

There have been evidences that MSP\textsubscript{19}-specific IgG2a antibody plays an important role in immunity against malaria infection (23). Firstly, our previous study demonstrated that immunization of mice with MSP\textsubscript{19} plus CpG ODN and Montanide ISA720 increased MSP\textsubscript{19}-specific IgG2a antibody titers 15 fold but did not alter the MSP\textsubscript{19}-specific IgG1 antibody titers,
compared to the immunization with MSP19 in Montanide ISA720 alone. After infection with
P. yoelii YM mice immunized with MSP19 plus CpG ODN and Montanide ISA720 were
completely protected with no parasitemia detectable over 24 days of observation while mice
immunized with MSP19 in Montanide ISA720 alone succumbed infection and all died with high
parasitemia (13). Secondly, transferring immune sera depleted of IgG2a could not confer
protection in recipient mice following P. chabaudi infection but the immune serum depleted of
IgG1 could (26). However, the mechanism of MSP19-specific IgG2a antibody in mediating
protection is not certainly known. IgG2a subclass prefers to bind FcγRI and then mediate
antibody-dependent cell-mediated cellular cytotoxicity (ADCC), antibody-dependent cellular
inhibition (ADCI) and phagocytosis but the MSP19-specific IgG2a antibody has been reported
not to use the Fc function for antibody-mediated protection (21,28). It may function by blocking
parasite invasion or inhibiting MSP1 processing which is required for erythrocyte entry (2,31).

In summary, we have demonstrated that MSP19 immunization formulated with CpG
ODN and Montanide ISA51 induces long term antibody response and confers complete
protection against blood-stage malaria parasite infection. Secondly, MSP19-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). Taken together, the combination of Montanide ISA51
and CpG ODN adjuvants are encouraged to use in clinical trial.

Acknowledgements

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 for AM Krieg for a gift of CpG ODN 1826 and SEPPIC, France for Montanide ISA51. We are indebted to Drs. Michael F Good and Michelle Wykes for critical reading the manuscript.

References


Figure 1. Levels of MSP19-specific antibody responses two weeks after complete immunization of BALB/c mice with PBS or MSP19 plus CpG ODN/ISA or CFA/IFA. (A) O.D. values of IgG antibody in sera diluted at 1/1,000,000. (B) Total IgG antibody titers. (C) IgG1 and IgG2a antibody titers. Data show mean ± S.E. Four mice were used in each experiment. Symbols represent individual mice in each group.
Figure 2. Duration of MSP1\textsubscript{19}-specific total IgG antibody responses after immunization. BALB/c mice were immunized with PBS or MSP1\textsubscript{19} plus CpG ODN/ISA or CFA/IFA. Sera were collected monthly and assayed for antibody titer by ELISA. Data show geometric mean ± S.E. of four mice in each group.
Figure 3. MSP1\textsubscript{19}-specific antibody subclass responses over 12 months after complete immunization. BALB/c mice were immunized with PBS or MSP1\textsubscript{19} plus CpG ODN/ISA or CFA/IFA. Sera were assayed at 6 and months after immunization for IgG1 and IgG2a antibody subclasses by ELISA. Data show O.D. values of IgG1 (dilution 1/100,000) and IgG2a (dilution 1,000) \pm S.E. Four mice were used in each group.
Figure 4. Parasitemia of BALB/c mice challenged with *P. yoelii*YM-pRBC 12 months after complete immunization. Groups of four BALB/c mice were immunized with PBS or MSP119 plus CpG ODN/ISA or CFA/IFA and then challenged with $1 \times 10^4$ *P. yoelii* YM-pRBC 12 months later. Data show parasitemia of individual mice. ¶, death.