DURATION OF THE ANTIBODY RESPONSE TO THE 19 KILODALTON FRAGMENT OF *PLASMODIUM YOELII* MEROZOITE SURFACE PROTEIN-1 ($MSP1_{19}$) FOLLOWING IMMUNIZATION USING CpG OLIGODEOXYNUCLEOTIDE AND MONTANIDE ISA51 AS ADJUVANT IN MICE

PIMMADA JEAMWATTANALERT

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ABSTRACT

Merozoite surface protein-1 (MSP1) is a leading malaria vaccine candidate and can induce protective immunity in mice and monkeys. However, how long the MSP1-specific antibody response lasts is not known. This study aimed to investigate the duration of this immune response. Groups of BALB/c mice were immunized for four doses or one dose with $MSP1_{19}$ mixed with CpG oligonucleotide (ODN) and Montanide ISA51 or with Complete/Incomplete Freund's adjuvants (CFA/IFA). By four-dose immunization, $MSP1_{19}$-specific antibody levels in mice immunized with $MSP1_{19}$ in CpG ODN/Montanide ISA51 persisted at a high level over 12 months after the last immunization while those in mice immunized with $MSP1_{19}$ in CFA/IFA decreased gradually and were lower significantly at the 12^{th} month than those at the first month after the last immunization. Both groups were still protected against *P. yoelii* infection after challenge at month 12 but the protection of the former group was better than that of the latter one as observed by lower patent parasitemia. The isotype of $MSP1_{19}$-specific antibody was investigated and found that CpG ODN/Montanide ISA51 enhanced both IgG1 and IgG2a antibody levels, but it promoted the increase of IgG2a antibody titer much more than that of IgG1 antibody titer compared to CFA/IFA. More interestingly, a single-dose immunization was as efficient as the four-dose immunization in induction of protective $MSP1_{19}$-specific antibody response, as demonstrated by antibody titer and protection against *P. yoelii* challenge infection. The peak of antibody response was maximum by 4 weeks and comparable to that of antibody response induced by four-dose immunization. However, the antibody level declined gradually over 22 weeks of observation. Taken together, these findings indicate that protective immunity to $MSP1_{19}$ lasts long following immunization with its repeats.

KEY WORDS: $MSP1_{19}$/ MALARIA/ ANTIBODY/ CpG OLIGODEOXYNUCLEOTIDE / MONTANIDE ISA51

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ระยะเวลาการตอบสนองของแอนติบอดีต่อโปรตีนน้ำหนักโมเลกุล 19 กิโลดาลตัน บนส่วนผิวของเมอโรซอยท์ ($MSP1_{19}$) ของเชื้อพลาสโมเดียม โยอิลิอายหลังจากฉีดกระตุ้นภูมิคุ้มกันโดยใช้ CpG oligodeoxy-nucleotide และ Montanide ISA51 เป็น Adjuvant ในหนูทดลอง
(DURATION OF THE ANTIBODY RESPONSE TO THE 19 KILODALTON FRAGMENT OF *PLASMODIUM YOELII* MEROZOITE SURFACE PROTEIN-1 ($MSP1_{19}$) FOLLOWING IMMUNIZATION USING CpG OLIGODEOXY-NUCLEOTIDE AND MONTANIDE ISA51 AS ADJUVANT IN MICE)

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บทคัดย่อ

โปรตีนบนผิวเมอโรซอยท์-1 (MSP1) เป็นโปรตีนที่อยู่ระดับแนวหน้าที่จะพัฒนาเป็นวัคซีนมาลาเรีย การศึกษาในหนูทดลองและลิงพบว่า โปรตีนชนิดนี้สามารถกระตุ้นภูมิคุ้มกันในการป้องกันการติดเชื้อมาลาเรียได้ อย่างไรก็ตามการตอบสนองของแอนติบอดีที่จำเพาะต่อ MSP1 มีระยะเวลานานเท่าใดยังไม่ทราบแน่ชัด ดังนั้น การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาระยะเวลาการตอบสนองของภูมิคุ้มกันดังกล่าว โดยการฉีดกระตุ้นภูมิคุ้ม กันในกลุ่มหนูทดลองสายพันธุ์ BALB/c ด้วย $MSP1_{19}$ ผสมกับ CpG ODN/Montanide ISA51 หรือ CFA/IFA จำนวน 4 ครั้ง หรือ 1 ครั้ง ผลการทดลองพบว่าในการฉีดกระตุ้นหนู 4 ครั้งด้วยโปรตีน $MSP1_{19}$ ผสมกับ CpG ODN/Montanide ISA51 มีระดับแอนติบอดีที่จำเพาะต่อ $MSP1_{19}$ สูงคงที่ นานถึง 12 เดือน ในขณะที่หนูทดลองที่ถูกกระตุ้นด้วยโปรตีน $MSP1_{19}$ ผสมกับ CFA/IFA มีระดับแอนติบอดีที่ลดลง อย่างช้าๆและต่อเนื่องตลอดเวลา 12 เดือน และเมื่อเปรียบเทียบระดับแอนติบอดีของเดือนที่ 12 และเดือนที่ 1 พบว่าลดลงอย่างมีนัยสำคัญ หนูทั้งสองกลุ่มยังคงสามารถป้องกันการติดเชื้อ *P. yoelii* ได้ แต่ในกลุ่มที่ได้รับ โปรตีน $MSP1_{19}$ ผสมกับ CpG ODN/Montanide ISA51 ป้องกันได้ดีกว่ากลุ่มที่ได้รับโปรตีน $MSP1_{19}$ ผสมกับ CFA/IFA ซึ่งสังเกตได้จากปริมาณเชื้อในเลือดต่ำกว่าในช่วงติดเชื้อ การศึกษาชนิดของ แอนติบอดีที่จำเพาะต่อโปรตีน $MSP1_{19}$ พบว่า การใช้ CpG ODN/Montanide ISA51 ให้ผลในการ กระตุ้นการสร้างแอนติบอดีทั้งชนิด IgG1 และ IgG2a ได้ดีกว่าการใช้ CFA/IFA แต่ CpG ODN/ Montanide ISA51 ส่งเสริมการสร้าง IgG2a เพิ่มมากกว่ามากกว่า IgG1 ที่น่าสนใจอย่างหนึ่งในการศึกษา ครั้งนี้ พบว่า การฉีดกระตุ้นภูมิคุ้มกันด้วย $MSP1_{19}$ เพียงครั้งเดียวมีประสิทธิภาพเทียบเคียงกับการฉีดกระตุ้น 4 ครั้งโดยเปรียบเทียบระดับแอนติบอดีและการป้องกันการติดเชื้อ *P. yoelii* การตอบสนองของระดับแอนติบอดี เพิ่มขึ้นถึงระดับสูงสุดภายใน 4 สัปดาห์ และระดับแอนติบอดีนี้เท่าเทียมกับระดับแอนติบอดีที่ได้จากวิธีการฉีด กระตุ้น 4 ครั้ง อย่างไรก็ตามระดับแอนติบอดีค่อยๆลดลงภายใน 22 สัปดาห์ที่ศึกษา ดังนั้นผลการศึกษานี้แสดงให้ เห็นว่าภูมิคุ้มกันต่อ $MSP1_{19}$ ในป้องกันการติดเชื้อมาลาเรียสามารถอยู่ได้ยาวนานหลังการฉีดกระตุ้นด้วยโปรตีน $MSP1_{19}$ และฉีดกระตุ้นซ้ำๆ

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CONTENTS

CONTENTS (Cont.)

LIST OF TABLE

LIST OF FIGURES

LIST OF FIGURES (Cont.)

LIST OF ABBREVIATIONS

ADCI	antibody-dependent cellular inhibition
AdPyCS	a recombinant adenovirus expressed *P.yoelii* circumsporozoite protein
APC	antigen presenting cells
ARF	acute renal failure
AS ONs	antisense oligonucleotides
ATN	acute tubular necrosis
BCG	bacillus calmette-guerin
BSA	bovine serum albumin
° C	degree (s) Celcius
CD	cluster of differentiation
CFA	complete Freund's adjuvant
CO_2	carbondioxide
CpG	cytosine-phosphate-guanosine
CTLs	cytotoxic T lymphocytes
CMI	cell-mediated immunity
CSP	circumsporozoite protein
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
E. coli	*Escherichia coli*
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular receptor kinases
G-6-PD	glucose-6-phosphate dehydrogenase
GPI	glycosylphosphatidylinositol
h	hour
H_2SO_4	sulfuric acid
ICAM	intercellular adhesion molecule

LIST OF ABBREVIATIONS (Cont.)

id	intradermally
IFA	incomplete Freund's adjuvant
IFN	interferon
Ig	immunoglobulin
IL	interleukin
im	intramuscularly
in	intranasally
iNOS	inducible nitric oxide synthase
ip	intraperitoneally
iv	intravenously
KAHRP	knob-associated histidine-rich protein
kDa	kilodalton
kg	kilogram(s)
LPS	lipopolysaccharide
MAPKs	mitogen-activated protein kinases
μl	microliter(s)
mg	milligram(s)
MHC	major histocompatibility complex
MSP	merozoite surface protein
NK cell	natural killer cells
NMS	normal mouse serum
ODN	oligodeoxynucleotides
OPD	o-phenylenediamine
PAMPs	pathogen associated molecular patterns
PBS	phosphate buffer saline
PDCs	plasmacytoid dendritic cells
P. falciparum	*Plasmodium falciparum*
po	per-orally
pRBC	parasitized red blood cells

LIST OF ABBREVIATIONS (Cont.)

P. yoelii	*Plasmodium yoelii*
RDS	respiratory distress syndrome
RTS	region T-cell epitopes the hepatitis B surface antigen
sc	subcutaneously
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SIRS	systemic inflammatory response syndrome
TBVs	transmission blocking malaria vaccines
TCR	T cell receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
VacPyCS	a recombinant vaccinia virus expressed *P.yoelii* circumsporozoite protein
WHO	World Health Organization

CHAPTER I
INTRODUCTION

1.1 Rationale and Statement of the Problem

Malaria is still a serious health problem in tropical countries, with an estimated 300-500 million clinical cases and 2.7 million deaths per year (1). The causative agent of malaria is *Plasmodium* spp. This parasite has a very complex life cycle. Even though there has no malaria vaccine licensed, many malaria vaccine candidates have been on clinical trials, including merozoite surface protein-1 (MSP-1).

MSP-1 is a leading malaria vaccine candidate involving in malaria parasite invasion of erythrocytes. In *Plasmodium falciparum*, MSP-1 is synthesized as a large precursor on the surface of merozoite. Proteolytic cleavage of MSP-1 leaves a C-terminal 19 kDa fragment ($MSP1_{19}$) on the surface of the parasite (2, 3). $MSP1_{19}$ is a highly conserved and is composed of two epidermal growth factor (EGF)-like domains that contain protective epitopes. Immunization with recombinant $MSP1_{19}$ of *P. falciparum* or *P. yoelii* formulated with adjuvants produced from yeast or as a Glutathione-S-transferase (GST) fusion protein produced from bacteria can protect monkeys or mice, respectively, against challenge infection. Protection has been correlated, in the mouse model, with high level of $MSP1_{19}$-specific antibodies that are predominantly of the IgG1 and IgG2b isotypes following immunization using Complete/Incomplete Freund's adjuvant (CFA/IFA) (4). Importantly, MSP-1 is expressed in other *Plasmodium* species and the processing that generates $MSP1_{19}$ appears to be similar, enabling the various malaria parasites and models for the study of $MSP1_{19}$ induced protection (5, 6).

More effective adjuvants for use in human are likely to be necessary for effective vaccine against malaria and other diseases (7). An immunological adjuvant has been used for more than six decades, to accelerate, prolong, and enhance the quality of specific immune response to an immunogen when incorporated. For many years, the only adjuvants available for general use in human vaccines have been

aluminum salts. The need for new adjuvants becomes clear with the advent of purified recombinant and synthetic peptide vaccine candidates, and with the realization that alum adjuvants are poor inducers of Th1 and cytotoxic T cell responses (8).

The protective formulation of vaccines has included bacterial DNA. Bacterial DNA and synthetic oligodeoxy-nucleotides (ODN) containing unmethylated CpG dinucleotides can induce murine B cells to proliferate and secrete immunoglobulin *in vitro* and *in vivo* (9). The unique difference of bacterial DNA from the others is that bacterial DNA contains unmethylated CpG motifs in the appropriate frequency of 1 in 16 while vertebrate and plant DNA contains methylated CpG motifs at a frequency of 1 in 4 and is generally flanked by bases which constitute immune-neutralizing rather than immune-stimulatory motifs (10). The ability of CpG ODNs to directly induce professional antigen-presenting cells (APCs) such as DCs to secrete cytokines such as IL-12, TNF-α and IFN-γ are critical in their striking enhancement of cellular immune response (11). The potent immune activation by CpG ODNs has implications for the design and to search for a delivery system that is appropriate as constituent of vaccine for human use. Recent studies have shown that CpG ODN enhances immune response to $MSP1_{19}$ and prone to induce more Th1 response (12). However, there is no elucidation whether the immune response lasts long in protection against malaria parasite infection. We also ask whether $MSP1_{19}$ vaccine formulation with CpG ODN can induce protection immune response if a single immunization is performed, and how long its lasts.

The aim of the present study is, therefore, to investigate the duration of antibody responses induced by a standard four-dose and single-dose immunization of mice with $MSP1_{19}$ using CpG ODN in Montanide ISA51 as adjuvants.

1.2 Objectives

1.2.1 General Objective

To investigate the duration of the immune response to *P. yoelii* $MSP1_{19}$ following immunization with the antigen using CpG ODN#1826 and Montanide ISA51 as adjuvant in mice.

1.2.2 Specific Objectives

1.2.2.1 To determine the level of antibody response to $MSP1_{19}$ in mice following immunization with *P. yoelii* $MSP1_{19}$ mixed with CpG ODN#1826 and Montanide ISA51.

1.2.2.2 To determine the duration of antibody response to $MSP1_{19}$ in mice following immunization with *P. yoelii* $MSP1_{19}$ mixed with CpG ODN#1826 and Montanide ISA51.

1.2.2.3 To investigate the protective immune response against *P. yoelii* infection following long term immunization with *P. yoelii* $MSP1_{19}$ mixed with CpG ODN#1826 and Montanide ISA51.

1.3 Hypothesis

$MSP1_{19}$-specific immune response can be induced and lasts long following immunization with $MSP1_{19}$ mixed with CpG ODN#1826 and Montanide ISA51.

CHAPTER II
LITERATURE REVIEW

2.1 Impact of Malaria

Malaria is a tropical disease of major global health significance. There are approximately 300 to 500 million cases each year and around 2.7 million deaths (1). At-risk groups include those in whom immunity has not yet developed, i.e., travelers, young children in endemic areas and those in whom immunity has diminished , i.e., people and pregnant women from endemic areas who exposed to infection. The result of malaria diseases as impediment to economic and social development in endemic regions. Malaria is considered a re-emerging disease, due largely to the spread of drug-resistant parasite strains, poverty of health-care and difficulties in implementing and maintaining vector control programs in developing countries. The disease is high appearance in sub-Saharan Africa, and is common throughout tropical regions of China, India, Southeast Asia, and South and Central America. *Falciparum* malaria causes the most morbidity which occurs in children under the age of ten, and a severe in under the age of five. Protecting these children from malaria is a major goal of current malaria vaccine development efforts (13,14).

2.2 Etiology of Malaria

Malaria is caused by several species of the parasite *Plasmodium.* There are approximately 156 named species of *Plasmodium* which infect various species of vertebrates. Four are known to infect humans; *P. falciparum, P. vivax, P. malariae* and *P. ovalae.*

Malaria is transmitted via the bite of an infected female *Anopheles sp* mosquitoes. The resulting diseases in human can devastating. After spreading rapidly through the bloodstream to the liver, the parasite emerges again into the bloodstream, finally to settle in the red blood cells, where it multiplies and emerges in bursts of new organisms. These parasites can cause particular damage to the nervous system, liver,

and kidney. In young children and adults who have not recently been infected (and have not developed natural immunity), which can result in death within hours from cerebral malaria. Others die later in the infection from anemia or liver and kidney failure. Untreated, up to 20% of persons infected with *falciparum* malaria will die (15).

2.3 Life Cycle of Malaria Parasite

Malaria parasite has a complex life cycle (Figure1). When an infectious female mosquito feeds on an individual, parasites (called sporozoites) are injected into the subcutaneous tissue, and directly into the blood steam. The *P. falciparum* life cycle includes a non-pathogenic, symptomless extraerythrocytic stage, which is followed by the invasion of mature erythrocytes by infective forms (merozoite) and the initiation of pathogenic intraerythrocytic stages. During the extraerythrocytic stage sporozoites invade hepatocytes, in which they replicate asexually for a period of 5 to 10 days for the human malaria species. Each sporozoite produces tens of thousands of merozoites per infected hepatocyte, that will initiate the intraerythrocytic stages of the infection. The erythrocytes contain mature schizonts, and at the time of rupture (48 h after erythrocyte invasion), each erythrocyte releases 15 to 30 merozoite progeny; these may bind to, and enter, uninfected erythrocytes to begin a new cycle. Some of these merozoites can be ingested by a mosquito during a blood meal. The sexual stages occur in the mosquito midgut lumen where a small number of gametocytes develop into mature ookinetes, some of which develop into oocysts after tranversing the midgut epithelium. At the later stages of infection, when the oocyst ruptures, only a fraction of the released sporozoites migrate to the salivary glands (16). Inoculation of sporozoites into the vascular of a new human host during mosquito feeding completes the malaria life cycle.

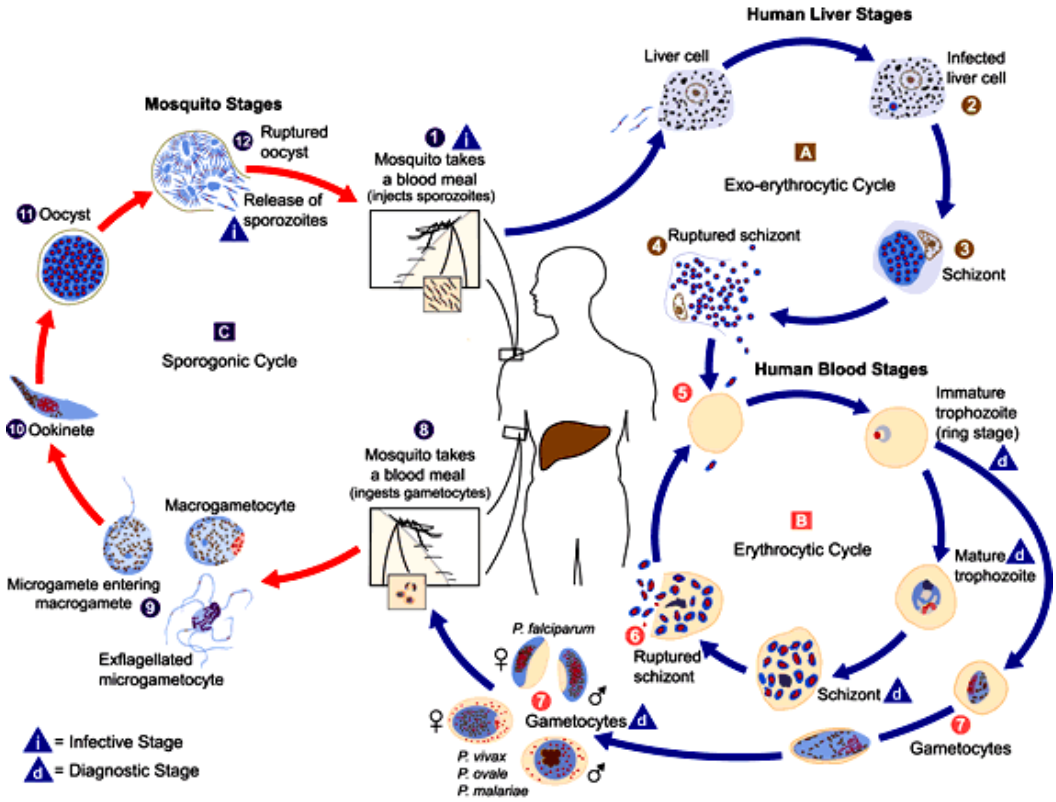

Figure 1. The life cycle of falciparum malaria parasite**.**

2.4 Pathology and Clinical Manifestations of Malaria

The sporozoite/liver stage represents the first encounter to the host. The parasite during the erythrocytic stage with the cyclic rupture of infected erythrocytes produces the clinical symptoms of malaria. Uncomplicated malaria the symptom can be rather non-specific and diagnosis can be missed. In people living where malaria transmission in intense which exposure to malaria from birth who survive childhood will have sufficient immunity to prevent clinical malaria, even though they might have parasites in their blood circulation all the time. Patients who have a history of malaria exposure with untreating can be accompanied by fever, chills, headache, myalgias, arthralgias, weakness, vomiting, diarrhea and cases which untreated infection continues for week or months can progress to severe malaria diseases including cerebral malaria, respiratory distress, anemia, thrombocytopenia and coagulation, renal failure and death (17).

2.4.1 Cerebral malaria

The main histopathological feature of cerebral malaria is widespread sequestration of infected erythrocytes in the cerebral microvasculature. Capillaries and postcapillary venule are dilated and congested and appear to be obstructed by parasitised erythrocytes. Minor endothelial cell damage is apparent and there is evidence of endothelial activation. Raised intracranial pressure has been reported in some children with cerebral malaria, but the contribution of elevated intracranial pressure to the pathogenesis of cerebral malaria remains unclear. Macroscopically, the brain appears oedematous and hyperemia, with evidence of haemazoin ('malaria pigment') deposition, and petechial punctate (or "ring") hemorrhages are appeared in non-immune children dying from cerebral malaria. Haemorrhage, vascular thrombi, ischaemia or inflammatory cell infiltrates is found in severe infected patients with plasmodium parasites (18).

2.4.2 Respiratory distress

Respiratory distress in individuals with severe malaria is well described and has been attributed to pulmonary oedema or to the adult respiratory distress syndrome, especially in adults. The pathogenesis of respiratory distress syndrome (RDS) in malaria is poorly understood, RDS can be caused by injury to the lung microvascular endothelium and alveolar epithelium via proinflammatory mechanisms involving activated neutrophils and/or cytokines (19). RDS in malaria can be caused by heart failure, parasite sequestration in the lungs, or the increased requirement for respiration associated with cerebral malaria (20). However, RDS is most often associated with metabolic acidosis, usually involving lactic acidemia (20), and this acidosis is a poor prognostic sign in children with severe malaria (21). The main clinical sign of RDS is deep breathing caused by attempts to release CO_2 to compensate for the acidosis. Lactic acidosis is likely to be the end result of reduced delivery of oxygen to the tissue and is exacerbated by anemia and hypovolaemia (22).

2.4.3 Anemia

Infection with *P. falciparum* causes change in the erythrocyte membrane, partly due to alteration of host membrane, and partly due to insertion of parasite

proteins. Electron-dense deposits (or 'knobs', that include the knob-associated histidine-rich protein, KAHRP) are associated with alterations in the erythrocyte membrane, which make red cells less deformable and presumably more susceptible to clearance. Exposure of novel immunoreactive antigens may lead to hemolytic anemia and accelerated splenic clearance but this dose not appear to be the major cause of anemia in chronic malaria (23). Cytokine-mediated suppression of hematopoiesis is likely to be a cause of dyserythropoiesis and explains why anemia in malaria is often disproportionately high compared with the level of parasitemia. This is supported by recent study, in which levels of IL-10 (a regulator of TNF) were significantly lower in patients with severe anemia than in other groups (24).

2.4.4 Thrombocytopenia and coagulation

Moderate thrombocytopenia is a common finding at presentation with all human malaria infections. It is unclear whether thrombocytopenia is caused mainly by decreased platelet survival, enhanced aggregation and sequestration from adherence to activated cells in the spleen and elsewhere, or antibody-mediated clearance. It is rarely associated with bleeding but may provide a clue to the diagnosis of malaria. Disseminated intravascular coagulation occurs in about 5% of patients with severe malaria. Microvascular thrombus formation can be seen in severe malaria, but is uncommon (25).

2.4.5 Renal failure

P. falciparum is the only species which causes acute renal failure (although *P. malariae* can cause a chronic nephropathy leading to nephritic syndrome and chronic renal failure). Sequestration of parasitised erythrocytes is evident in glomerular and interstitial vessels. There is also evidence of reduced renal blood flow and oxygen delivery in *P. falciparum* malaria. It is unclear whether this is a result of sequestration of parasitised erythrocytes in the kidney or a local or systemic effect of circulating vasoactive compounds. Malaria-associated ARF has features of acute tubular necrosis (ATN), seen in bacteria sepsis. Histologically there are changes in the tubules consistent with ATN, and haemoglobin tubular casts and tubular atrophy have been demonstrated in cases of black water fever. The descriptive term 'black water fever'

refers to a clinical setting in which the patient passes very dark urine as a result of excessive intravascular haemolysis, and is not necessarily associated with renal failure. It can occur with severe malaria alone but may be associated with administration of quinine or oxidant drugs in individual with glucose-6-phosphate dehydrogenase (G-6-PD) deficiency (13, 26).

2.4.6 Hypoglycemia

Hypoglycemia (blood glucose concentration < 2.2 mmol/l or 40 mg/dl) is generally associated with quinine infusion in adults and is most often attributed to quinine-induced hyperinsulinaemia, although other mechanism, such as the effect of circulating cytokines, are likely to be involved. In children, pretreatment of hypo-glycemia is important. It is present in 10-20% of African children on presentation with cerebral malaria and is associated with a poor prognosis. High parasitemias contribute to hypoglycemia and probably also to lactic acidosis. Parasites consume glucose at a rate of 70 times that of erythrocytes to generate energy from anaerobic glycolysis of glucose to lactic acid. Sick patients are also likely to have high levels of circulating cytokines, which also contribute to the abnormal metabolic state (17,18).

2.5 Immunity to Malaria

Immunity against the malaria parasite is complex and stage-specific. In different stages of the parasite's life cycle, only the asexual erythrocytic stage is associated with pathology and clinical disease. The parasite induces a specific immune response, stimulating the release of cytokines from human peripheral blood mononuclear cells (PBMC), which might play an important function in activating the host's monocytes, neutrophils, T cells, and natural killer (NK) cells to react to the subsequent liver and blood stage parasite (27).

Several antigens, specific to the liver stage have been identified and suggested that these antigens of the sporozoite present on the surface of infected hepatocytes in combination with MHC Class I and, resulting in recognition by cytotoxic T lymphocytes (CTLs) and killing of the infected cell, or stimulation of NK and $CD4^+$ T cells to produce interferon γ, which can trigger to the death of intracellular parasite (28). The plasmodium parasite developing within the host hepatocyte is the major

target of protective immunity at the extraerythrocytic stage. The CTLs may be directly cytolytic against malaria-infected hepatocytes by releasing perforin and granzyme or by binding to apoptosis-inducing receptors on the infected cells (29).

The merozoite enters the red blood cell by receptor-mediated endocytosis. At the time of erythrocyte rupture, parasite antigens are released into the bloodstream, stimulating the release of tumor necrosis factor α (TNFα) and other factors (30). Antibody binding to the surface of the merozoite, and to proteins that are externalized from the apical complex of organelles involved in erythrocyte recognition and invasion, seems to have an important role in immunity to asexual blood stages. This antibody could neutralize parasites, or lead to Fc-dependent mechanism of parasite killing by macrophages (31). The pathogenic manifestations during a malaria crisis are due to proinflammatory cytokines released by T cells and macrophages in response to malaria parasites and their products, including glycosylphosphatidylinositol (GPI) moieties, malaria pigment, and plasmodium-derived nitric oxide synthase (NOS)-inducing factor (27). Nitric oxide (NO), a molecule produced from L-arginine by the enzyme nitric oxide synthase (32), mediates a variety of biological functions including vascular and muscle relaxation, platelet aggregation, neuronal cell function, microbicidal and tumoricidal activity, and a range of immunopathologies (33).

2.5.1 Immunity to liver stage malaria

A pre-erythrocytic-stage vaccine represents one of the most interventions for prevention and control of malaria. Sterile protective immunity against challenge with *Plasmodium spp.* sporozoites can be induced in mice. Monkeys and humans after immunized with sporozoite that are attenuated by exposure to X-or γ-radiation. When sporozoites are experimentally irradiated, they are able to invade hepatocytes but are unable to mature to the stage that infects erythrocytes and clinical symptoms of disease and transmission of malaria do not occurs. The importance of immune mechanisms that are active in this irradiated sporozoite model is highlighted by the fact that infection-blocking immunity in humans rarely, if ever, occur under natural conditions. In the rodent model, immunization with heat killed, formalin inactivated or lysed sporozoites is not effective but protective immunity is induced in mice immunized with nonirradiated sporozoite and treated with chloroquine to prevent

erythrocyte infection (34). The importance of mechanisms is the requirement for live sporozoites targeting the liver. The infected hepatocyte was considered an immune-privileged site and pre-eyrthrocytic stage immunity was thought to be directed against the sporozoite in the circulation and to be mediated by antibodies, which neutralize the sporozoite infectivity for hepatocytes (14). *In vitro*, antigen-specific monoclonal antibodies could prevent the invasion and development of *P. falciparum* in human hepatocytes or eliminates *P. yoelii*-infected hepatocytes from culture (by direct sporozoite neutralization). *In vivo*, antibody mediated protective immunity against *P. berghei, P. yoelii, P. vivax* or *P. knowlesi* sporozoite challenge was demonstrated by passive immunization studies in mice and monkeys. However, since the extracellular sporozoites invade the hepatocyte with 2-30 minutes of inoculation, antisporozoite antibodies must be present in circulation at high titers and effort activity within minutes of infection. These antibody mediated protective immunity is unlikely to be completely effective (35). It is now generally accepted that the *Plasmodium spp.* parasite developing within the host hepatocyte is the major target of protective immunity directed against the pre-eryhtrocytic stage (14). Both $CD8^+$ and $CD4^+$ T cells recognize parasite-derived peptides presented by MHC Class I or Class II molecules, respectively, on the surface of infected hepatocytes. Nevertheless, protection against the pre-erythrocytic stage is mediated primarily by $CD8^+$ T cells (36). *In vivo* depletion of $CD8^+$ T cells failed to develop protection, adoptive transfer of $CD8^+$ T cells to naïve mice confers protection and mice which express no class I are not protected by active immunization or by adoptive transfer of wild-type, splenic T cells from immune mice (37).

For many years, protection against pre-erythrocytic stage malaria was presumed to be mediated directly by $CD8^+$ cytotoxic T lymphocytes (CTLs). However, cytokines and other factors (including nitric oxide) have also been involved (14). *In vitro* treatment of *Plasmodium spp.* infected hepatocytes with IFN-γ eliminated *P. berghei* or *P. falciparum* parasites from culture, *in vivo* administration of IFN-γ partially protected against sporozoite challenge with *P. berghei* in mice or *P. cynomolgi* in monkeys and *in vivo* depletion of IFN-γ failed to develop protective immunity induced by *P. berghei* sporozoites in mice. Furthermore, IFN-γ induced the production of nitric oxide *in vitro* and *in vivo* following *P. berghei, P. yoelii* or *P.*

falciparum sporozoite infection. The protection in mice induced by immunization with irradiated *P. berghei* or *P. yoelii* sporozoite is absolutely dependent on $CD8^+$ T cells, IFN-γ and nitric oxide that activated $CD8^+$ T cells to secrete IFN-γ, which subsequently activates iNOS (induced nitric oxide synthase) and induces the L-arginine-dependent nitric oxide pathway (38). Although the role of $CD8^+$ T cells is not clear in pre-erythrocytic-stage immunity in humans but it is likely to be involved.

A role of $CD4^+$ T cells in pre-erythrocyte-stage immunity came in early 1990s, when it was shown that *in vivo* depletion of $CD4^+$ T cells in irradiated sporozoite-immunized mice significantly reduced protective immunity (39). The result indicated that $CD4^+$ T cells were able to exert a direct inhibitory effect on the pre-erythrocytic stages of malaria parasites. Moreover, it was shown that a $CD4^+$ T cell clone derived from mice immunized with irradiated *P. berghei* sporozoites could confer immunity to naïve mice adoptively transferred with the clone and subsequently challenged with infective sporozoites (40). The protective effect of this clone, which recognizes a heretofore-unidentified antigen shared by both sporozoites and blood stages, provided the first direct evidence that $CD4^+$ T cells can directly mediate pre-erythrocytic-stage immunity. This activity of $CD4^+$ T cells was later confirmed in similar experiments using different $CD4^+$ T cell clones specific for epitopes of the *P. yoelii* CS protein. These CS-specific $CD4^+$ T cell clones were also capable of mediating protection to malaria when adoptively transferred into naïve mice (41). $CD4^+$ T cells also provide important helper functions required for other arms of the immune system to exert their anti-malaria effects. Recently, a study has shown that the development of neutralizing antibodies against the CS protein *in vivo*, both αβ T cells and MHC Class II molecules are required (42). Thus, $CD4^+$ T cells are essential for the induction of an optimal humoral response to malaria parasite.

2.5.2 Immunity to blood stage malaria

The mechanisms of merozoite invasion of the erythrocyte reveal immune evasion by the parasite and potential targets for vaccines. Invasion of erythrocytes occurs at the red blood cell (RBC) surface. The merozoite interacts with the RBC surface and returns the apical region towards the membrane (43). The contents of the apical organelles (rhoptries and micronemes) are expelled and a moving junction is

formed between the merozoite and RBC membrane. Then, the parasite enters a vacuole formed by invagination of the RBC membrane and membranes secreted from the parasite. Receptors that mediate invasion of RBCs by merozoites and invasion of liver by sporozoites are found in micronemes, on the cell surface, and in rhoptries. These are the major targets of neutralizing immune responses (43).

These receptors within organelles provide some protection to the parasite from antibody-mediated neutralization. The exposure to antibody occurs only between release from one RBC and invasion of another. Once inside RBC, some parasite molecules make their way to the RBC surface while others remain in membranous vacuoles within the cell (44). Since the RBC lacks Class I and II major histocompatibility complex (MHC) antigens, the blood-stage parasites are also protected from effector T cell response, which are dependent on Class I and II antigen-presentation pathways.

Immunity against blood-stage antigens occurs in both antibodies and T cells and in human, better evidence exists for antibody-mediated immunity. Epidemiological studies in malaria-endemic area support the idea that antibody response to blood-stage parasites is produced from naturally acquired immunity. Sera from humans living in hyperendemic area contain antibodies that prevent red cell invasion by targeting antigens on merozoites (45). A protective function of antibodies comes from passive transfer of purified immunoglobulins from 'naturally immune' individuals into partially immune children, which was found to produce rapid clearance of parasites in recipient children even when the antibodies did not block growth *in vitro* (46).

These sera contained high levels of antibodies to the variant of RBC surface antigen, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which would not kill *in vitro*, but would prevent sequestration of mature parasites *in vivo*, resulting in their splenic destruction. These results are consistent with the effector of anti-PfEMP1 antibodies which was induced in *Aotus* monkeys challenged with *P. falciparum* (47). In humans, sera from immune individuals have high titers of antibodies against PfEMP1 in addition to other surface and internal merozoite antigens, so investigators have speculated that PfEMP1 is a target for antibody-mediated immunity. Different domains of this erythrocyte surface-expressed antigen mediate cytoadherence of

parasitized RBC through interactions with specific receptors, for example, CD36 (on endothelial cell surfaces) and chondroitin surface A (on placental syncytio-trophoblasts) (48). PfEMP1 is encoded by the large and diverse *var* gene family, which plays a key role in clonal antigenic variation. Immune pressure results quickly in the emergence of parasites expressing different variant gene which that indicate some functional domains of the molecule may induce a degree of cross-variant immunity (49).

The direct effect of human antibody on parasites, *in vitro* incubation of infected erythrocytes with IgG from immune human donors and monocytes from a naïve donor kills the parasite within the RBC. This antibody-dependent cellular inhibition (ADCI) associated killing of the parasite is mediated by transferable soluble factors in macrophage supernatants (50). Cytophilic antibodies to merozoite antigens have been involved *in vitro* in assays with human macrophages and *in vivo* in SCID mice (51). ADCI may provide an alternative for antibody-mediated parasite clearance following vaccination.

Antibody-independent (cell-mediated), T-cell lines and clones can adoptively transfer protection, suggesting that $CD4^+$ T cells can control blood-stage parasites. Studies in animal model have involved antibody-independent mechanism in immunity against blood-stage malaria parasites which shown by clones and polyclonal of T cells (52). Speculation continues about the mechanisms underlying the $CD4^+$ T cell-mediated control of parasites. Cytokines and other direct effector mechanisms like nitric oxide and γδ T cells have been involved in parasite clearance (53). However, rodent studies should be extended to human malaria cautiously, because there are clear differences in the relative importance of antibodies and T cell effector function for parasite clearance between different rodent malaria species and hosts. While most of the evidence of T cells has come from murine studies, and studies in human have shown that repeated low grade infection induces sterile immunity in the absence of detectable antibodies suggests that T cell-mediated protection in human (54). Evidence from demonstrating that infection with some parasite species in rodents can lead to anergy and apoptosis of parasite-specific $CD4^+$ T cell (but not cells of other specificities) (55). This could be the malaria parasite strategy to inhibit host immunity. Other mechanisms of immune impairment by the malaria parasite have

been described, such as inhibition of dendritic cell maturation and activation by the parasite.

2.5.3 Immunological memory to malaria infection

Evidence for immunological memory has been recognized for more than 2,000 years (56). The observation that immunological memory to a virus can last a lifetime was first recorded in 1846 by Panum. Panum, a Danish physician, observed that elderly people on the Faeroes islands infected with the measles virus in 1781 were immune to measles epidemic that struck the island more than 65 years later in 1846 (57).

One role of the adaptive immune system is that it remembers previous encounters with antigen. Immune memory is very important in protection from infectious agents. Immune memory allows the host to initiate the secondary immune response on subsequent encounters with a pathogen. The secondary immune system to make a more vigorous (e.g., more antibody production) and a more effective response (i.e., higher affinity specific antibody) on re-exposure to previously encountered antigen. Long-term memory is thought to result from the presence of clonally expanded, antigen-specific B and T lymphocytes that persist in a resting state for many years and, in some cases, life-long.

The pathways of naïve T cells in taking to become memory T cells have been studied. Recent evidence suggests that naïve, effector, and at least two types of memory T cells exist in both mice and human (central-memory and effector-memory) (58). In humans, differentially spliced isoforms of the leukocyte common antigen (CD45) have historically been used to discriminate between naïve (CD45RA) and memory (CD45RO) T cells (59). However, more recent longitudinal studies examining $CD8^+$ CTL during the activation, apoptotic and memory phases of acute and chronic Herpes virus infections in humans have suggested that memory cells can also express CD45RA. Cycling $CD8^+$ T cells are CD27 positive, whereas terminally differentiated, perforin positive, CTL are CD27 negative, suggesting that the loss of CD27 expression may be a marker for effector and effector-memory CTL (60). In humans, T effector-memory (T_{EM}) and T central-memory (T_{CM}) cells differ both functionally and in their migratory properties and can be distinguished based on their

CD62L and CCR7 expression. T_{CM} cells express CCR7 and CD62L, whereas T_{EM} cells do not express CCR7 or CD62L. In both mice and humans, $CD4^+$ T_{CM} and $CD8^+$ T_{CM} cells reside within lymphoid organ. $CD4^+$ T_{CM} cells produce IL-2, IL-10, and divide rapidly upon restimulation, whereas $CD8^+$ T_{CM} cells retain their ability to secrete IL-2, but need to be re-primed to regain perforin killing effector function and secrete IFN-γ. $CD4^+$ T_{CM} cells are more responsive to TCR stimulation than naïve T cells and can also stimulate dendritic cells via CD40 ligand to produce IL-12. In contrast, $CD4^+$ T_{CM} cells reside in peripheral tissue to provide protection against reinfection, and upon TCR stimulation, rapidly produces IFN-γ or IL-4. $CD8^+$ T_{CM} cells also reside in peripheral tissue and exhibit immediate cytokine secretion and perforin killing activity (58).

The protective immunity in humans is based upon exposure to radiation-attenuated *P. falciparum* sporozoites (γ-spz). In contrast to naturally acquired protection that allows intermittent epitopes of infection to occur, γ-spz-induced protection is sterile and long lasting (61). After infectious γ-spz, γ-spz colonize the liver and undergo an abortive development into liver stage parasites that express an array of liver-and blood-stage antigen but do not induce clinical malaria (62).

Some studies have shown an association between protection induced by γ-spz and the emergence of memory $CD4^+CD45RO^+$ T cells. Memory $CD4^+$ T cells recognizes antigen expressed during liver-stage infection, and these include antigens present also on parasitized red blood cells (pRBC) (63). The memory $CD4^+$ T cells produce IL-4 upon *in vitro* recall with pRBC and most importantly, these evident in persons protected by γ-spz but not in γ-spz immune persons susceptible to malaria or malaria naïve persons. The possible contribution of IL-4 producing memory $CD4^+$ T cells includes help an antibody response, activation of effector T cells, and differentiation and possibly maintenance of the memory T cell compartment. Having established that induction of memory $CD4^+CD45RO^+$ T cells coincides with protection generated by immunization with *P. falciparum* γ-spz (63).

Recently studies about long-lasting protection against malaria of mice primed with a recombinant adenovirus expressing the circumsporozoite protein of *P. yoelii* (AdPyCS) and boosted a recombinant vaccinia virus expressing the same antigen

(VacPyCS) showed the increase levels of activated CS-specific $CD8^+$ and $CD4^+$ T cells, higher anti-sporozoite antibody titer, and greater protection in mice, when the time between priming and boosting with two viral vector was extended from 2 to 8 or more weeks. Most importantly, by using this immunization regimen, the protection of the immunized mice was found to be long lasting, namely complete resistance to infection of all animals for 3 months after priming. The results indicated that immunization with AdPyCS generates highly effective memory T and B cells that can be recalled long after priming by boosting VacPyCS (64).

In sub-Saharan Africa, congenital malaria is rare because few newborns develop clinical disease during the first few weeks of life (65). However, finding anti-*P. falciparum* immunoglobulin M (IgM) and IgE antibodies in cord blood of African newborns is not uncommon, as studies have reported parasite-specific IgM in up to 25.4% of cord blood samples (66). *P. falciparum* IgM in cord blood suggests that the fetus was infected in utero and B cell activation occurred. Alternatively, malarial antigens, perhaps as immune complexes, could have crossed the placental barrier and stimulated the response (67). The specificity of antimalarial antibodies produced by the fetus is unknown but may be limited, as the antigenic repertoire of fetal B cells less than adults. It is possible that a fetus may be able to produce antibodies to only a limited number of malarial antigens. It is important to determine the extent of in utero antigenic recognition because early immune priming may have a direct impact on the development of immunity to malaria when infants become infected later in life. Recently, King et al (68) studied acquired immune responses to *P. falciparum* MSP1 in the human fetus. These resulted that the memory B cells specific for the C-terminal region of *P. falciparum* merozoite surface proteins1_{19} ($MSP1_{19}$) were detected, a determinant involved in immune protection, in the cord blood of Kenyan newborns. Thus, exposure to malaria in utero or at the time of delivery could result in the induction of protection prior to primary natural infection.

A recent study on the duration of efficacy and immune responses following immunization with the *P. falciparum* circumsporozoite protein fused to the surface antigen of the hepatitis B virus (RTS, S) malaria vaccine has shown that responses fluctuated during the 6 month interval and unrecognized after second challenge with sporozoite and protection occurs in short duration. However, the findings point

maintaining immunologic memory and activated T cells which are protective to malaria parasite in the future (69).

Recently, Matsumoto et al demonstrated about long-lasting protective immunity against malaria parasites infection at the blood stage by recombinant live BCG (rBCG) secreting 15 kDa C-terminal region of merozoite surface protein-1 from *Plasmodium yoelii* ($MSP1_{15}$). BCG is alive vaccine and is able of surviving in antigen presenting cells, means that durable effects can be expected. These studies resulted that $rBCGMSP1_{15}$ can induce IFN-γ production until day 268 after booster immunization (70). However, the protective effects are not sufficient and have short time, and most deaths from severe malaria are seen in children less than 5 years.

2.6 Malaria Vaccines

Vaccination against *P. falciparum* is the intervention that is most likely to reduce malaria-associated severe morbidity and mortality in infants and young children in area with the most intense transmission and to reduce the risk to non-immune travelers to endemic areas. The rationale for developing a malaria vaccine includes prevention to infection (pre-erythrocytic vaccine), prevention of disease (blood-stage vaccine) and reduction of transmission (transmission blocking vaccines). The general strategy since the cloning of *P. falciparum* blood-stage antigens in 1983 has been to develop subunit vaccines composed of defined antigens that can be synthesized chemically or by recombinant technology. As malaria immunity is stage-specific, the main focus in the development of a subunit vaccine has been to identify critical target antigens at each stage of the life cycle. This has involved the characterization of components of the parasite that are essential for its survival or development, as well as a detailed investigation of the immunological mechanisms that confer partial protection following natural infection. A range of different vaccine delivery methods has also been developed. These include malaria antigens linked to strong T-helper epitopes, synthetic malaria peptides or recombinant proteins formulated with adjuvant liposome or other particles, malarial protein gene sequences incorporated into live vectors, such as the attenuated vaccines virus strain, NYVAC and DNA vaccine.

2.6.1 Pre-erythrocytic vaccines

Pre-erythrocytic vaccine development initially focused on inducing antibodies to the sporozoite surface. Antibodies directed to the circumsporozoite protein (CSP) repeat [asparagines (N), alanine (A), proline (P), NANP] were shown to neutralize sporozoite *in vitro* but to give inconsistent protection against *P. falciparum* infection *in vivo*. It is likely that the failure of the early CSP vaccine was due to a failure to induce cell-mediated effector mechanisms, such as cytotoxic T lymphocytes and cytokine-mediated inhibition of parasite development.

In 1994, studies reported that immunization of mice with plasmid DNA could protect against a complex parasite pathogen. Results showed that immunization of mice with plasmid DNA encoding a pre-erythrocytic stage *P. yoelii* antigen, PyCSP, induced antigen-specific $CD8^+$ cytotoxic T lymphocyte (CTL) and antibody responses and protection against sporozoite challenge. Moreover, the levels of $CD8^+$ CTL and $CD8^+$ interferon (IFN)-γ responses induced by DNA immunization were greater than the levels induced by protective immunization with irradiated sporozoites (71).

Although no immune correlation of protection was identified, antibodies and $CD4^+$ T cells may be involved. The technology of DNA vaccines demonstrated that vaccination with plasmid DNA encoding the *P. falciparum* CSP induced antigen-specific, genetically restricted, $CD8^+$ CTL in malaria-naïve human which in this study, protective efficacy was not assessed (72).

In 1998, development of a vaccine was designed to induce protective immune responses against pre-erythrocytic stage malaria, the RTS, S vaccine-comprising the *P. falciparum* circumsporozoite protein (CSP) fused to hepatitis B surface antigen, formulated in adjuvant. Results showed that six out of seven volunteers were protected but this protection was of short duration (69).

Recently, RTS, S/AS02A were studied by the University of Barcelona in Mozambique reported that over a 6-months period in children 1-4 years of age, RST, S/AS02A conferred 29.9% efficacy for first clinical episoded and 57.7% efficacy for severe malaria due to *P. facipalum* (73).

2.6.2 Erythrocytic stage vaccines

People in endemic areas develop clinical immunity despite frequent blood-stage infections that are usually asymptomatic, demonstrating that it is not necessary (and may not be desirable) to prevent infection but in order to prevent disease. Based on the principle of mimicking immunity acquired in an endemic area, a blood-stage vaccine could be designed to reduce or interfere with parasite replication, and numerous animal studies have confirmed that immunization induces protection that may be more effective than chronic repeated infections. A vaccine could also be designed to prevent pathology, rather than infection. An 'anti-adhesion' vaccine could prevent a key step in pathogenesis, for example, to prevent malaria in pregnancy that appeared to be associated with chondroitin sulphate A binding. An 'anti-disease' vaccine could neutralize key toxins, such as glycosylphosphatidylino-sitol (GPI), responsible for pathology or passively transferred antibody could potentially neutralize harmful cytokines. Recent work identifying GPI of *P. falciparum* as a toxin capable of inducing massive cytokine release provides the basis for a vaccine that could induce immunity to clinical malaria, thus mimicking earliest immunity developed by individuals living in an endemic area.

In recent years many studies have been conducted with pre-erythrocyte and blood-stage vaccine for specific vaccine candidates. Merozoite surface proteins are a major focus of research for blood-stage vaccines and candidates for future trials are MSP1 (in the form of $MSP1_{19}$ or $MSP1_{42}$) and AMA1. Other merozoite proteins (MSP3, MSP4, MSP5 and RAP2 and GLURP) are at an earlier stage along the vaccine development path. Many clinical trials in humans have been performed using blood-stage antigen alone or in combination with pre-erythrocytic antigens (74). The most have been conducted in humans with SPf66, a combination of erythrocytic antigens that has shown mixed results, making it difficult to justify more trials. Amid vaccines based on blood-stage antigens, a mixture of three combinant blood-stage antigens, *P. falciparum* RESA and fragments of two merozoite surface proteins ($MSP1_{190L}$, consisting of the relatively conserved blocks 3 and 4 of MSP1 fused with a universal T-cell epitope derived from the circumsporozoite protein of *P. falciparum*, and the near full-length MSP2 sequence of the 3D7 cloned line) formulated in an oil-based adjuvant. This vaccine has undergone human trials and although there was no

reduction in episodes of clinical malaria, a significant reduction in parasite density was observed (75).

The trial of a vaccine based on $MSP1_{19}$ (FVO and D7 strains of *P. falciparum*) fused to $CD4^+$ T cell epitopes from tetanus toxoid concluded that the vaccine were immunogenic but had a sufficiently high rate of adverse reactions to warrant alternative formulation (76). Moreover, trials are planned for vaccine based on the C-terminus of $MSP1_{19}$ and AMA1 vaccine comprising two allelic forms (clones 3D7 and FVO). A chimeric molecule that includes $MSP1_{19}$ and AMA1 is likely to be tested in the future (77). Thus the several recombinant blood-stage vaccines will undergo safety and immunogenicity studies in malaria endemic area in the future.

The Merozoite Surface Protein-1_{19} ($MSP1_{19}$**)** is a leading candidates for a vaccine targeted at the erythrocytic stage of plasmodial parasite development. Recently, there has been increasing interest in this polypeptide, particularly in the carboxyl-terminal EGF-like domains (78). The MSP-1 antigen is synthesized as a large precursor (190-230 kDa, depending upon the parasite species) and is subsequently processed into four fragments. The final processing of the C-terminal 42-kDa fragment yield a 33-kDa protein, which is shed, and a relatively conserved 19-kDa part ($MSP1_{19}$), which remains attached to the merozoite during erythrocyte invasion and is expressed by the parasite during the early ring stages (79). The C-terminal fragment, which contains a number of a cysteine residues conserved across species boundaries, is retained on the merozoite surface. A large body of evidence indicates that MSP-1 is a target of protective immune responses, primarily antibody-mediated, in both rodent and primate malaria models. Protection was high titer antibodies that recognized a conformation epitope, as evidenced by abolition of protection by reduction and alkylation of protein (80). Both antibodies and effector T cells appear to play important roles in the protection of mice against *P. yoelii.* However, the protective immunity induced by vaccination with recombinant *P. yoelii* $MSP1_{19}$ is mediated largely by antibodies (4, 81). Passive transfer of $MSP1_{19}$–specific antiserum or monoclonal antibodies (MAb) into naïve mice suppressed an otherwise lethal *P. yoelii* infection, whereas vaccination with $MSP1_{19}$ failed to protect mice deficient in immunoglobulin μ-chains (4). In contrast, immunization with defined $MSP1_{19}$-derived T cell epitopes does not induce protective immunity and transfer of T

cell lines from $MSP1_{19}$–immunized mice dose not confer protection to naïve recipients (82). Nevertheless, a function $CD4^+$ T cell response is a prerequisite for the generation of potent humoral response vaccination with protein antigens (4). The immunity is mediated in the mouse model by high level of $MSP1_{19}$–specific antibodies that are predominantly of the IgG1 and IgG2b isotypes at the time of infection, but not with effector $CD4^+$ T cells nor other accessory factors associated with cell-mediated immunity, e.g., macrophages, nitric oxide and an intact spleen (4, 12).

The antibody response to the $PfMSP1_{19}$ in groups of subjects living in areas of Brazil with different levels of malaria transmission was studied. The prevalence and the levels of IgG to $PfMSP1_{19}$ increased with the time of exposure and were positively correlated with the absence of clinical symptoms in parasitemic patients. The IgG1 isotype was higher among subjects with long-term exposure to unstable malaria transmission and in asymptomatic infection (83).

2.6.3 Transmission blocking vaccines

Transmission blocking malaria vaccines (TBVs) against malaria are to induce immunity against the stages of the parasites which infect mosquitoes by reducing of interrupting the transmission of malaria in human and mosquito population. Action of malaria TBVs serves to prevent the escape of the parasites from a human host, if used in combination with vaccines against the liver and blood-stage parasites, serves to prevent or reduce the spread of parasites which become resistant to such vaccines and potential to great prolong the effective life of other malaria vaccines (84).

An understanding of the biology and epidemiology of malaria transmission is important to vaccines development. Because malaria depends on transmission between a human population and appropriate species of Anopheles mosquito, malaria transmission is strictly limited to distances of the aqueous breeding site of these mosquitoes. For most practical purposes a human population must lie within a perimeter of about half to 1 km in radius of such a breeding site for active malaria transmission to take place by effective transmission blocking measure, for example, drainage, larvicing, residual insecticide spraying, and including TBVs (85). Thus for malaria control, TBVs is a local and a focal matter. This means that sensible and

effective decisions on malaria TBVs deployment can, and should be made on the basis of individual small local communities.

Transmission blocking immunity can be induced by immunization of the vertebrate host with the extra-cellular male and female gametes of malaria parasites, with other sexual and mosquito midgut stage of the parasites, namely the zygotes and ookinetes. Transmission blocking immunity is mediated by antibodies against surface protein antigens of these tages and acting within the midgut of a blood fed mosquito. The antibodies against the gametes act within 5-10 min after the ingestion of the blood to prevent fertilization or to destroy the gametes and newly fertilized zygotes in complement-mediated reactions. Neutralizing and cytophilic antibodies are active up to 12-24 h later against the developing and mature ookinetes of the malaria parasite as they prepare to penetrate the mosquito midgut (84).

Recent studies showed that molecules such as the chitinase of the malaria ookinete plays an essential role in the ookinete's passage across the mosquito midgut (86). However, the target antigens developed are surface proteins of the extra-cellular male and female gametes, zygote and ookinete stages of the parasites.

The *P. falciparum* and *P. vivax* forms of Ps25 molecules, respectively Pfs25 and Pvs25, have been successfully expressed in yeast in immunogenic form. The material has been used to generate immune sera in mice which have potent transmission blocking activity against gametocytes of the corresponding species of *Plasmodium* (86).

The constructs for the Ps25 formulations used in the preliminary tests and in the human trials clearly have antigenicity close to that native molecules that they represent and as shown by their ability to induce strong transmission blocking antibody responses in the animal systems tested. Further trials with clinical grade materials and formulations of both *P. falciparum* and *P. vivax* Ps25 molecules are in preparation. These will involve the testing of adjuvants other than alum and the preparation of different constructs representing the Ps25 molecules (87).

2.7 Adjuvant

Immunological adjuvant may be defined as any substance that, when incorporated into a vaccine formulation, acts generally to accelerate, prolong, or enhance the quality of specific immune response to vaccine antigens. Adjuvant mechanisms of action include the followings; first, functioning as a depot to increasing the biological or immunologic half-life of vaccine antigen, second, improving antigen delivery to APCs as well as antigen processing and presentation by the APCs, finally, inducing the production of immunomodulatory cytokines (88).

Immunologic adjuvants have been under development and test for most century. In 1926, Glenny demonstrated the adjuvant activity of aluminum compounds with use of an alum-precipitated diphtheria toxoid vaccine (89). In the mid 1930s, Freund developed immunologic adjuvant composed of a water-in-mineral-oil emulsion and containing mycobacteria as an additional immunomodulator. This adjuvant is known as Complete Freund's adjuvant (CFA), which one of the most effective adjuvants but cannot be used in human vaccines because it is highly reactogenic. However, Incomplete Freund's adjuvant (IFA), which does not contain mycobacteria is used in several HIV vaccines under clinical evaluation and licensed in the United Kingdom for influenza vaccine (90).

The mechanisms of adjuvants action are poorly understood, which must be chosen for use with a particular vaccine responses desired. The first mechanism of adjuvant action identified was the so-called depot effect, in which gel-type adjuvants, such as aluminum hydroxide, or emulsion-based adjuvants, such as Incomplete Freund's adjuvant, associate with antigen and facilitate transport of antigen to the draining lymph node, where immune responses are generated. Immunogenicity of small antigens such as synthetic peptides that otherwise would be rapidly cleared from the injection site and from draining lymph node can be improved by the use of adjuvants that form particles or otherwise associate with and hold antigen (88).

Adjuvants can increase cellular infiltration, inflammation, and trafficking to the injection site, particularly for antigen presenting cells (APCs); promoting the activation state of APCs by upregulating costimulatory signals or MHC expression; enhancing antigen presentation; or inducing cytokine release for indirect effects (91).

The immune response to injected vaccine antigen has shown that two major subsets of $CD4^+$ T cells (Th1 and Th2) have been identified in mouse and human, based on their differ cytokine patterns. Th1 responses are typically characterized by the induction of delayed-type hypersensitivity response, and secretion of IFN-γ, IL-2 and IL-12. In contrast Th2 responses are characterized by the induction of circulating or secretory antibodies and the secretion of IL-4, IL-5, IL-6 and IL-10. The different cytokine secretion patterns are mutually antagonistic, and upregulation of one type of response normally results in downregulation of the alternative. In mice the production of the IgG2a antibody isotype is widely recognized as characteristic of a Th1 response, whereas a Th2 response is associated with the induction of IgG1 (92).

Although different adjuvants may induce comparable levels of functional antibodies, the respective cytokine profiles and antibody isotype may differ. In certain situations, the type of response induced (Th1 or Th2) may have a significant impact on the protective efficacy of a vaccine (91).

Adjuvants enhance Th1 responses with induction of IFN-γ and delayed-type hypersensitivity, leading to the production of IgG subclasses that fix complement and bind with high affinity to Fc-γ-I receptors (e.g., IgG1 in humans and IgG2a in mice) (93). These IgG subclasses are most active in complement-mediated lysis and in antibody-dependent cell-mediated cytotoxicity effection mechanism.

Immune protection following vaccination depends predominantly on the generation of immunologic memory, mediated by B and T lymphocytes of the acquired immune system, which have highly restricted antigen specificity. Vaccines are effective to prevention of infection, or to prevention of disease. Antibodies are most important for prevention of infection, but difficult to receive with some pathogens e.g., HIV. In contrast, T cells are mainly responsible for recognizing and killing infected cells and function to limit the spread of infection. For most pathogens, immune response can successfully eradicated initial infections before the infection is innately self-limiting. However, some pathogens are difficult to clear e.g., HIV and HCV. Potent adjuvants and novel vaccine strategies may be required to permit the successful elimination.

The dominant pattern in immunology for immune system develops to discriminate self from nonself. Results progress in understanding the clonal

recognition of antigenic epitopes mediated by B and T lymphocytes. However, the self/nonself framework offers into why some nonself antigens are poorly immunogenic. In the last decade, alternative models of immunity have been established, which the selective pressures on the host induce a pro-inflammatory innate immune response to pathogen associated molecular patterns (PAMP's) and tissue damage. These more ancient responses are not antigen-specific and are mediated by the innate immune system. These new models of immunity, vaccines will elicit a potent immune response only when the nonself antigens mimic key aspects of infectious agents or cause some degree of localized tissue damage.

Vaccines such as bacterial toxoids and attenuated viral vaccine are sufficiently potent to induce protective immune responses. In contrast, recombinant vaccines are highly purified, lack many of original pathogen and do not evoke strong immune responses. Therefore, vaccine adjuvants are critical to make the vaccine enough immunogenic. So, the role of vaccine adjuvants for recombinant vaccines is to ensure that the vaccine likeness infection closely, is enough to initiate a potent immune response. In addition, the innate immune system directs the balance of humoral and CMI, and adjuvant can control the type of acquired immune response induced (94).

2.8 Cytosine-phosphate-Guanosine Containing DNA (CpG DNA)

The polyanionic polymeric structure of nucleic acids creates the potential for biological macromolecules. Nucleic acids are generally very poor antigens, and do not readily induce immune responses against themselves. However, recent studies have shown that bacterial genomic DNAs, can activate a broad range of immune cells, including macrophages, natural killer (NK) cells, B cells and dendritic cells (DCs) (95). The immune stimulatory effects of bacterial DNA are now known to be due to a subtle structural difference from vertebrate DNA. In vertebrate DNA, the CpG dinucleotide is suppressed in frequency to about one quarter of the expected level, is 80% methylated at the 5-position of the cytosine, and is usually flanked by a cytosine on the 5′ side and/or a guanine on the 3′ side (10). In contrast, bacterial DNA is unmethylated and typically has a near random distribution of CpG dinucleotides and flanking bases. The immune stimulatory effects of bacterial DNA may be mimicked by synthetic ODNs containing CpG dinucleotides, in particular, base contexts termed

CpG motifs, but these effects are abolished if the CpG dinucleotides are methylated (9). The least immune stimulatory base contexts for CpG dinucleotides are those in which a cytosine is on the 5′ side and/or a guanine is on the 3′ side.

2.8.1 Role of CpG DNA in induction immunity defenses

2.8.1.1 Stimulation of cells of innate immune system

CpG DNA also has direct stimulatory effects on murine and human monocytes, macrophages and DCs, which up-regulate the expression of costimulatory molecules, and secretes IL-12 and other cytokines and chemokines. The IL-12 and other pro-inflammatory cytokines secreted by these adherent cells have a costimulatory effect on human and murine NK cells, enhancing the production of IFN-γ, as well as their ability to lyse target cells. Although T cells are not directly stimulated by CpG DNA, the production of IFN-α and other pro-inflammatory cytokines contributes to T cell activation, while the enhanced expression of costimulatory molecules facilitates the presentation of antigen and the induction of specific T-cell responses, especially those of the Th1-type (96).

a. Monocyte

Although only relatively few studies have used monocytes, human monocytes isolated from peripheral blood were found to transcribe large amounts of IFN-α, IFN-β, and IL-12 within 3 h of incubation with pDNA containing immuno-stimulatory sequence (97). Human monocytes also secreted IL-12, IL-1, IL-6, and TNF-α when exposed to CpG sequences. Bacterial DNA and CpG ODNs stimulated IL-6, TNF-α, and up-regulated intercellular adhesion molecule-1 (ICAM-1) on monocytes.

b. Macrophages

Murine macrophages respond rapidly to stimulatory CpG motifs by secreting IL-12 and TNF-α (98). The secreted IL-12 can elicit IFN-γ from NK cells, which can further activate macrophages. In particular, IFN-γ stimulated macrophages can be induced by DNA containing immunostimulatory CpG sequences to up-regulate

the expression of the antimicrobial inducible nitric oxide synthase, or iNOS. While the activation state of macrophages is enhanced towards antimicrobial activity by CpG sequences, it appears to be downregulated with respect to antigen processing. Thus, for example, treatment of macrophages with stimulatory CpG ODN results in the decreased synthesis of MHC II and antigen processing (99).

c. Dendritic cells

Like macrophages, dendritic cells (DCs) are stimulated to secrete cytokines in response to CpG; they appear to be extremely sensitive, and secrete high levels of both IL-12 and IL-18 (100). However, in contrast to the effects of stimulatory CpG motifs on macrophages, the antigen presenting ability of DCs appears to be enhanced by these molecules. Thus, CpGs stimulate the up-regulate of MHC II as well as co-stimulatory molecules such as CD40 and B7-2 on immature DCs. Stimulatory CpG sequences (as well as LPS) induce DC maturation in addition to stimulation (101). These mature DCs are capable of T cell activation. The CpG-induced immune stimulatory effects appear to depend on the derivation of the DC. Thus, for example, while monocyte-derived DCs can produce IL-12 and IL-18 in response to CpGs, they do not up-regulate co-stimulatory molecules like primary DCs (102).

d. NK cells

In vivo, the introduction of bacterial DNA or CpG-containing ODN have been seen to result in a rapid (<4 h) induction of IFN-γ and the generation of IFN-γ forming cells (identified as NK cells) in the spleen. The activation of NK cells by CpG motifs is thought to be largely indirect, occurring through the actions of Th1 cytokines such as IFN-α, IFN-β, TNF-α, and IL-12, coming from CpG activated monocytes, macrophages, DCs, and possibly B cells. Activation results in an increase of the lytic activity of NK cells and their secretion of IFN-γ. Producing of IFN-γ may in fact result from synergy between the action of IL-12 and a direct interaction of the stimulatory DNA within the NK cells (103).

2.8.1.2 Stimulation of adaptive immune system

Several different immune cell types are directly or indirectly activated by CpG DNA. CpG DNA directly induces B cells to proliferate, differentiate and to secrete several immune regulatory cytokines as well as immunoglobulin (9). CpG DNA also interferes with the process of programmed cell death, or apoptosis, in B cells. These stimulatory effects are not limited to mouse B cells, but are also seen in human B cells. B cell activation by CpG DNA shows strong synergy with signaling through the B cell receptor, which should tend to promote the development of antigen-specific immune responses against foreign antigens encountered at the same time as the DNA (96).

a. B cells

A large body of evidence on isolated B cells demonstrates that the effects of CpG stimulatory motifs on B cells are direct in nature. These motifs are mitogenic and immune stimulatory for B cells, e.g., MHC II as well as the co-stimulatory molecules B7-1 and B7-2 is all up-regulate as a consequence of CpG stimulation. In addition, activation of B cells by CpG motifs appears to synergize with signaling through the B cell receptor, implying that the result of humoral responses will be antigen specific. Stimulation also results in the secretion of IL-6 and IL-10, as well as promotes the production of immunoglobulin (97).

b. T cells

Although initial studies pointed towards the direct activation of T cells by immunostimulatory DNA sequences, subsequent studies have shown that stimulation is indirect, and is more likely an end result of cytokine products from other cells, e.g., IFN-α, IFN-β. Indirectly then, CpG DNA induces T cells to up-regulate B7-2 and CD69 (97).

2.8.2 Signaling pathways activated by CpG DNA

There is some controversy concerning whether the pattern recognition receptors (PRRs) for CpG DNA is a cell surface molecule or is intracellular. Based on experiments demonstrating that CpG ODN immobilized on a solid support lost their

immunostimulatory effects, it was suggested that cell uptake is required for immune stimulation (9). In contrast, other investigations have reported that CpG ODN immobilized on sepharose beads retained their immune stimulatory effects, suggesting that the CpG receptor is a cell-surface molecule. However, it now appears that CpG ODN linked to sepharose beaded can still be taken up by cells, and that if CpG ODN are truly immobilized, they are not immune stimulatory. Other evidence supporting an intracellular location for the CpG PRR is that transfection of CpG ODN into cells enhances their effects, and that drugs which interfere with the endosomal uptake mechanisms of CpG ODN, such as chloroquine and quinacrine, completely and specifically block CpG-induced immune activation (104). Structure-activity studies have identified more potent antagonists of CpG ODN, but the mechanism of action of these antagonists has not yet been completely elucidated.

ODN containing CpG motif activates host defense mechanisms leading to innate and acquired immune responses. The recognition of motifs required Toll-like receptor (TLR) 9, while triggers alterations in cellular redox balance and the induction of cell signaling pathways including the mitogen activated protein kinases (MAPKs) and NF kappa B cells that express TLR-9, which include plasmacytoid dendritic cells (PDCs) and B cells, produce Th1-like proinflammatory cytokines, interferons, and chemokines (105).

Mitogen-activated protein kinases (MAPKs) have important roles in transducing many signals in lymphocytes and other cells. Two of the MAPK pathways, the p38 kinases and the c-Jun NH_2-terminal kinases (JNK) are activated within 10 min in B cells exposed to CpG DNA. In macrophages, the extracellular receptor kinases (ERK) are also activated upon CpG exposure (106). Activation of these pathways is required for the downstream events induced by CpG DNA, including activation of transcription factors and increased expression of cytokines, protooncogenes and anti-apoptotic genes. Although the overall effects of CpG DNA have some similarities to immune stimulation mediated by LPS, the molecular pathways involved differ (96).

TLR have recently emerged as key receptor responsible for recognizing specific conserved components of microbes including lipopolysaccharides from Gram-negative bacteria, CpG DNA, and flagellin. Full activation of inflammatory responses

by TLR may require the assembly of receptor signaling complexes including other transmembrane proteins that may influence signal transduction. TLR may be additional receptors participating in innate recognition of microbes, and recent studies demonstrate strong interactions between signaling through these receptor and signaling through TLR (107).

2.8.3 Th1-like immune activation by CpG DNA

CpG DNA activates macrophages and DCs to express increased levels of costimulatory molecules, and to produce a variety of chemokines and cytokines, especially Th1-like cytokines, such as IL-12, TNF-α and IFN-γ. NK cells are activated to produce IFN-γ, which feeds back on the macrophages and DCs to further stimulate them in a Th1-like direction. B cells exposed to CpG DNA have some counter-regulatory effects due to the production of IL-10, but nevertheless contribute to the overall immune stimulatory reaction by expressing increased levels of the costimulatory molecules B7-1 and B7-2, and producing IL-6 and immunoglobulin. Although CpG DNA dose not directly activate resting T-cells, it creates a Th1-like cytokine environment that tents to promote the differentiation and function of Th1 cells and suppress that of Th2 cells (96).

2.8.4 Role of CpG DNA as a vaccine adjuvant

Genetic or DNA vaccines are an exciting new approach to immunization, which has recently been reviewed. Since DNA vaccines are grown in bacteria, their CpG motifs would be unmethylated. Engineering DNA vaccines by increasing the number of stimulatory CpG motifs and decreasing the number of inhibitory sequences can significantly enhance their efficacy, but small changes in the numbers of CpG motifs in DNA vaccines may have no impact. Nevertheless, the addition of vector DNA containing CpG motifs to a DNA vaccine enhances the response, showing the contribution of the added CpG motifs (108). In fact, it is now clear that DNA vaccines only transfect a small proportion of the DCs in responding lymph nodes to express the encoded antigen, but the CpG motifs in the DNA vaccine activate a much larger fraction of the DCs, creating an enhanced environment for generation of immune responses. DNA vaccines may be considered to have two essential components. First,

the antigen encoded in the vector is expressed, providing the specific antigen which is the target of the response. Second, the CpG dinucleotides in the plasmid act as a built-in adjuvant, triggering antigen-presenting cells in the vicinity to secrete pro-inflammatory cytokines and to present newly acquired antigens more effectively. The CpG motifs of the DNA vaccine increased the expression of MHC Class II, CD40, CD80, CD86 in DC as well as increasing the production of IL-12, a pro-inflammatory cytokine or Th1 cytokine. It is also reviewed by Coombes and Mahony (109) that these changes affectively lead to an increase in DC migration to lymph nodes, as well as enhanced activation of $CD8^+$ T cells. This 'pro-inflammatory' priming by CpG containing regions most probably acts as an adjuvant of the DNA vaccine, skewing the immune system towards a Th1 style response, which favours cell-mediated killing via $CD8^+$ T cells. This may also explain why results of DNA vaccine experiments using 'Gene guns' to deliver the vaccine, coated on micro-particles, injected intradermally, resulted in a stronger Th2, or humoral response, bias than vaccines administered intramuscularly. In the words, as the Th1 response is not primed by CpG motif recognition by immune cells upon DNA vaccine delivery, the immune response is bypassing a step necessary for a Th1 bias, resulting in an environment favouring the evolution of a Th2 or humoral response (9).

By creating a Th1-like cytokine environment, by strongly activating antigen presenting cells, and by acting in synergy through B and T cell antigen receptors, CpG DNA appears to have tremendous potential as a general vaccine adjuvant for inducing antigen-specific Th1-like immune responses. In mice, CpG DNA is a more potent adjuvant for inducing strong Th1-like T and B cell responses than the gold standard, complete Freund's adjuvant (110). CpG DNA is also an effective adjuvant for difficult antigens, including peptides and thymus-independent antigens. CpG DNA is an effective adjuvant for tumor vaccines, where it shows particular synergy with another adjuvant, GM-CSF. The adjuvant effects of CpG DNAs are enhanced by formulation with alum or other adjuvants that may act as a depot. As a role, the CpG DNA and the antigen must be administered in the same anatomic. Presumably, this is because of the need for the same APC to be activated and also to present the antigen. CpG DNA has been shown to be an effective adjuvant through multiple routes of administration, including intravenously (iv), intramuscularly (im), intradermally (id),

subcutaneously (sc), intranasally (in) and orally (po) (111). The ability of CpG DNA to induce systemic and mucosal immune responses following vaccination through a mucosal route is especially noteworthy, since this has been a major barrier in effective vaccination. As many pathogens enter the host through mucosal surfaces, the ability of CpG DNA provide protection at this surface may be of great value.

In 2003, Hirunpetcharat et al showed that mice immunized with $MSP1_{19}$ formulated with synthetic CpG ODN 1826 and Montanide ISA were protected against *P. yoelii* injection with no parasites detected in the blood. The CpG OND 1826 significantly enhanced both IgG1 and IgG2a antibody responses in Montanide ISA51-adjuvanted mice (12).

In 2004, the CpG ODN 1826 in the $yMSP1_{19}$ plus ISA51 vaccine contributed towards the induction of higher levels of IgG2a and IgG2b (Th1 type) antibodies, that CpG ODN 1826 caused a shift towards a Th1 type of immunse responses that could be responsible for the higher degree of protection immunity (112).

2.8.5 Toxicology of CpG DNA

Activation of innate immunity with CpG DNA unleashes a two-edged sword. On the good side, immune activation has utility in multiple therapeutic settings as reviewed above. On the other hand, excessive or inappropriate immune activation can cause toxicity. An example of an extreme form of immune toxicity is the systemic inflammatory response syndrome (SIRS), which is the cause of death in septic shock. Although it is less effective at inducing this than endotoxin, repeated administration of high dose of CpG DNA alone or in combination with LPS or hepatoxin can induce SIRS (113, 114). A milder example of immune toxicity from a potent immune stimulatory drug such as CpG DNA would be the induction of an autoimmune disease. As an immune stimulatory DNA molecule, it seemed especially likely that administration of CpG DNA could trigger the development of systemic lupus erythematosus, an autoimmune disease characterized by autoimmunity to DNA and the production of antibodies to double stranded DNA (115). However, animal studies have demonstrated that administration of CpG DNA dose not trigger lupus in normal mice, but actually reduces lupus severity in mice genetically predisposed to develop this. Nevertheless, administration of IL-12 has been shown to promote the

development of autoimmunity both in experimental models and in human subjects. Since CpG DNA produces the induction of large amounts of IL-12, it may be expected to have similar effects in some settings. Indeed, CpG DNA can activate autoreactive Th1 effector cells specific for the central nervous system protein, myelin basic protein, which can trigger the development of experimental autoimmune encephalomyelitis (EAE) in susceptible mice (116).

CHAPTER III
MATERIALS AND METHODS

3.1 Study Design

This research was an experimental study, using inbred BALB/c mice with the same range of age and sex.

3.2 Mice and Parasites

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

3.3 Recombinant $MSP1_{19}$ Protein

Recombinant $MSP1_{19}$ protein of *P. yoelii* was produced from *Saccharomyces cerevisiae* as a FLAG-fusion protein (FLAG-$MSP1_{19}$) and purified as described by Sakkhachornphop *et al*. (117). Briefly, the yeast was cultured in Yeast extract-Peptone-Dextrose (YPD) agar, incubated at 30°C for 2-3 days, cultured in Yeast extract-Peptone expression media (YPEM) liquid medium with shaking at 250 rpm, 30°C for 24 h, and transferred in Yeast extract-Peptone 4 high stability expression media (YPHSM) with shaking at 250 rpm, at 28°C for 48 h. Supernatant was collected, dialysed with 0.85% normal saline for 3-4 days, and filtered through a 0.22 μm millipore filter. Finally, the supernatant was purified by passing through an anti-FLAG M1 antibody agarose gel column. The purity of the recombinant protein was judged by SDS-PAGE as a single band with molecular weight of 19 kilodaltons (117).

3.4 Adjuvants

CpG ODN#1826 (TCCATGACGTTCCTGACGTT) kindly provided by Dr. Sathit Pichayangkul, Armed Forces Research Institute of Medical Sciences, Bangkok, was used. Complete Freund's adjuvant (CFA) and Incomplete Freund's adjuvant (IFA) were obtained from Sigma Co, Louis. MO, USA.

3.5 Immunization of Mice with $MSP1_{19}$

For four-dose immunization, groups of five mice were immunized with 20 μg $MSP1_{19}$ or PBS which was mixed with 50 μg CpG ODN#1826 in Montanide ISA51 in a volume of 100 μl via s.c., s.c., i.p., and i.p. injections on day 0, 21, 42 and 56, respectively (4). Others were immunized s.c. with the same dose of $MSP1_{19}$ or PBS which was mixed with CFA on day 0 and then s.c., i.p., and i.p., on day 21, 42 and 56, respectively, with $MSP1_{19}$ or PBS mixed with IFA (4).

For single-dose immunization, groups of five mice were immunized s.c. with 20 μg $MSP1_{19}$ or PBS mixed with 50 μg CpG ODN#1826 in Montanide ISA51, or 20 μg $MSP1_{19}$ or PBS mixed with CFA in a volume of 100 μl.

3.6 Plasma Collection

Twenty microliters of blood was collected from mouse-tail vein into 180 μl heparin/PBS (10 μl of 5,000 IU/ml heparin in 1 ml of PBS). The blood was centrifuged at 2,000 rpm for 5 minutes. The plasma was collected into a new tube and stored at –20°C until used. For four-dose immunization, blood was collected one day before each immunization, 14 days after the last immunization and then every month for 12 months. For single-dose immunization, blood was collected one day before immunization and then every week for 22 weeks.

3.7 Antibody Assay by ELISA

A 96–well plate was coated with 100 μl of $MSP1_{19}$ (0.5 μg/ml) overnight at 4°C. After being washed three times with 0.05% Tween 20 in PBS (PBS-T), wells were blocked with 200 μl of 0.5% BSA/PBS and the plate was incubated at 37°C for 1 h. The solution was discarded and 100 μl serially diluted plasma was added. The

plate was incubated at 37°C for 1 h. After being washed five times with PBS-T, the plate was added with 100 μl of 1/3,000 horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG. For isotype determination, wells were washed and added with 100 μl of 1/3,000 HRP-conjugated anti-mouse IgG1 or IgG2a antibody. After being incubated at 37°C for 1 h, wells were washed five times and added with 100 μl OPD substrate solution (H_2O_2+o-phenylenediamine in citrate-phosphate buffer pH 5.0). The plate was incubated for 30 min in the dark at room temperature before adding 100 μl 1N H_2SO_4 and read for optical density (O.D.) at 490 nm., using an ELISA reader. $MSP1_{19}$-immune serum and serum from unimmunized mice were used as positive and negative controls, respectively.

3.8 Challenge of Mice with *P. yoelii* YM-pRBC.

Mice were infected i.p. with 1 x 10^4 live *P. yoelii* YM-pRBC. Parasitemia was monitored daily by microscopic examination of blood smears stained with Giemsa reagent.

3.9 Statistical Analysis

The statistical significance of O.D. or titer between the experiment and control groups was analyzed using Student's *t* test of Sigma Plot window version 4.0 (SPSS) program.

CHAPTER IV
RESULTS

1. Kinetics of $MSP1_{19}$-Specific Antibody Response in Mice Following Four-Dose Immunizations with *P. yoelii* $MSP1_{19}$ Mixed with CpG ODN#1826 and Montanide ISA51

The IgG antibody response to $MSP1_{19}$ was determined in BALB/c mice immunized for four doses with $MSP1_{19}$ mixed with CpG ODN#1826 and Montanide ISA51. Blood was collected and tested by ELISA on the day just before each immunization and days 14 and 28 after the last immunization. As can be seen in Figure2A, the antibody level modestly increased after the second immunization (mean O.D. ± S.E. at plasma dilution of 1/1,000,000; 0.579±0.096), then more rapidly increased 14 days after the last immunization (mean O.D. ± S.E.; 1.260±0.168), and remained at high level over 28 days after the last immunization (mean O.D. ± S.E.; 1.306±0.241).

In contrast, mice immunized with $MSP1_{19}$ mixed with CFA/IFA produced the $MSP1_{19}$-specific antibody more rapidly after the second immunization and then remained at high level until 28 days after the last immunization, i.e., the mean O.D. ± S.E. at plasma dilution of 1/100,000 at days 41, 55, 70 and 84 were 1.344±0.384, 1.317±0.403, 1.228±0.386, and 1.739±0.246, respectively (Figure 2B).

Control groups of mice immunized with PBS mixed with CpG ODN#1826/ Montanide ISA51 or CFA/IFA did not produce $MSP1_{19}$-specific antibody (Figure 2A, B).

2. The Efficacy of CpG ODN#1826/Montanide ISA51 in Induction of $MSP1_{19}$-Specific Antibody Response

To compare the efficacy of $MSP1_{19}$-specific antibody production between using CpG ODN#1826/Montanide ISA51 and CFA/IFA which has been used as a gold standard adjuvant in immunization of mice, blood collected at 14 days after the last

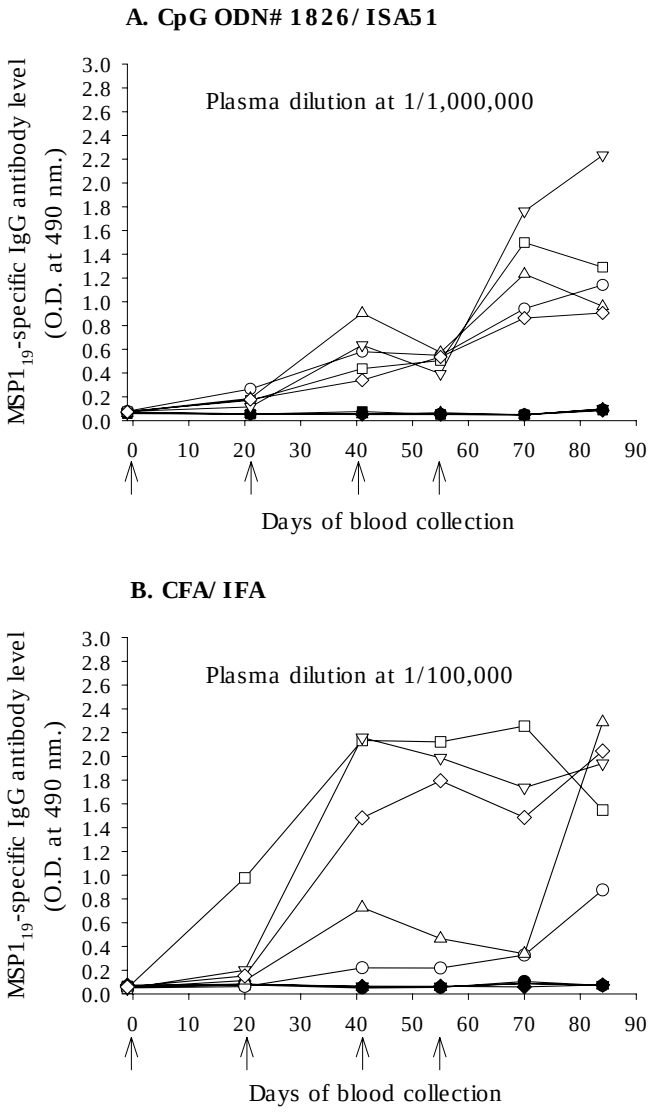

Figure 2 Kinetics of antibody response to $MSP1_{19}$ in mice following four-dose immunization with $MSP1_{19}$ formulated in CpG ODN#1826/Montanide ISA51 or CFA/IFA adjuvants. Five BALB/c mice were immunized with $MSP1_{19}$ (opened symbols) or PBS (closed symbols) mixed with CpG ODN#1826/Montanide ISA51 (A) or CFA/IFA (B) on day 0, 21, 42, and 56 as described in the Materials and Methods. Plasma was collected and made a dilution of 1/1,000,000 (A) and 1/100,000 (B) and then assayed for $MSP1_{19}$-specific IgG antibody by ELISA. Each line/symbol represents each mouse. Arrows represent the points of time of immunization. One line represents one mouse.

immunization was dilution 1/1,000,000 and assayed for $MSP1_{19}$-specific antibody by ELISA. As can be seen in Figure 3, the $MSP1_{19}$-specific IgG antibody level in CpG ODN#1826/ Montanide ISA51 adjuvanted mice was much significantly higher than that in CFA/IFA adjuvanted mice as demonstrated by O.D. level (mean O.D. ± S.E.; 1.260±0.168 and 0.351±0.044, respectively, p=0.0008) (Figure 3A), and also by the mean antibody titer (mean log IgG antibody titer ± S.E.; 7.204±0.445, and 6.401±0.160, respectively, p=0.0052) (Figure 3B).

3. Kinetics of $MSP1_{19}$-Specific Antibody Response in Mice Following A Single-Dose Immunization with *P. yoelii* $MSP1_{19}$ Mixed with CpG ODN#1826 and Montanide ISA51

It was interesting to ask whether a single-dose immunization with $MSP1_{19}$ can induce antibody responses comparable to a standard four-dose immunization. To address this question, mice were s.c. injected only one time at day 0 with $MSP1_{19}$ mixed with CpG ODN#1826/Montanide ISA51 or CFA. The IgG antibody specific for $MSP1_{19}$ was determined in plasma collected every week after the immunization. The antibody was able to be detected 14 days after immunization and reached to maximum level at day 28 in the same manner in both CpG ODN#1826/Montanide ISA51 and CFA adjuvanted immunizations (mean O.D. ± S.E., 2.551±0.119 and 2.042±0.304), respectively, (Figure 4A and B). The antibody levels were sustained until day 42 in either group of $MSP1_{19}$-immunized mice adjuvanted with CpG ODN#1826/Montanide ISA51 or CFA/IFA (Figure 4).

4. Comparison of $MSP1_{19}$-Specific IgG Antibody Response Between A Single-Dose and Four-Dose Immunizations

As the rising of $MSP1_{19}$-specific antibody following a single-dose and four-dose immunizations was in a similar manner, the levels of the antibody in both immunizations were compared. Using CpG ODN#1826/Montanide ISA51 as adjuvant, the mean log antibody titers of single-dose immunized mice at day 28 and 63 following immunization were 6.806±0.296 and 6.833±0.382 (mean log antibody titer ± S.E.) which were not different from that of four-dose immunized mice at day 70 since the initial immunization (7.203±0.445; p=0.1351 and p=0.1953, respectively)

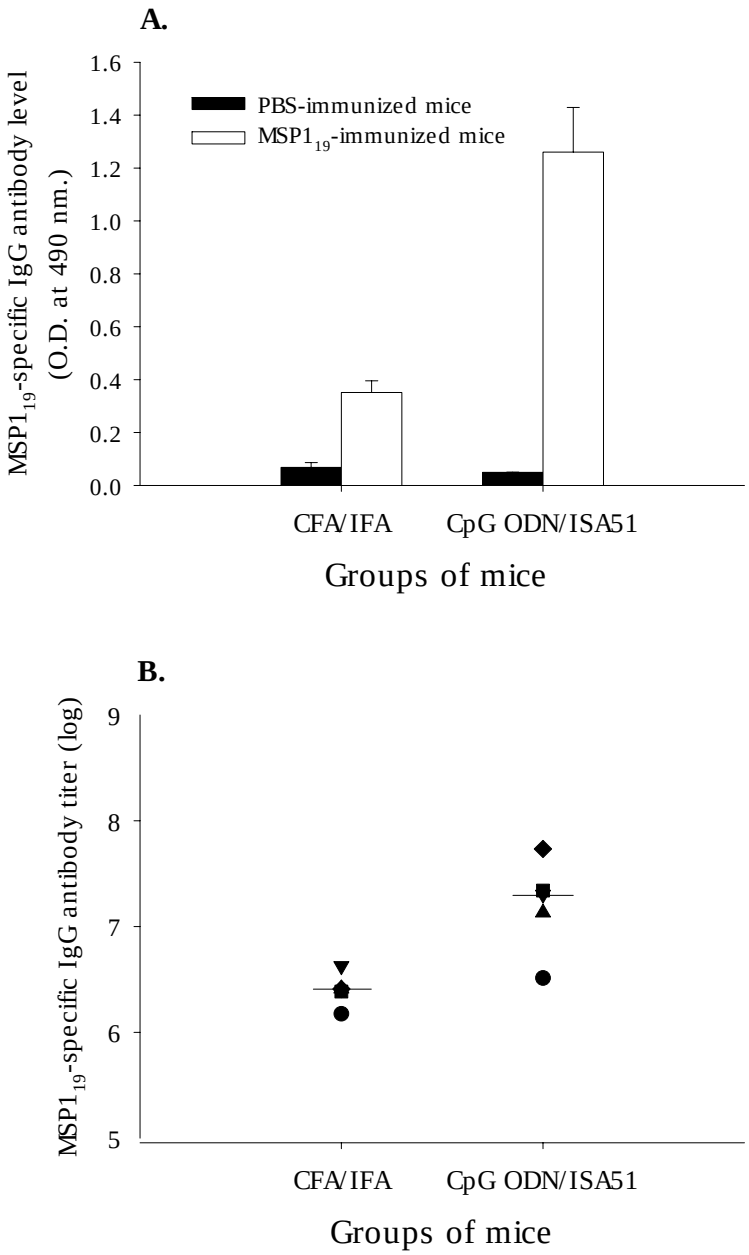

Figure 3 Comparison of the efficacy of MSP1$_{19}$-specific antibody production between using CpG ODN#1826/Montanide ISA51 and CFA/IFA as adjuvant in MSP1$_{19}$ immunization of mice. The antibody levels were shown as O.D. (A) and titer (B). One symbol represents one mouse.

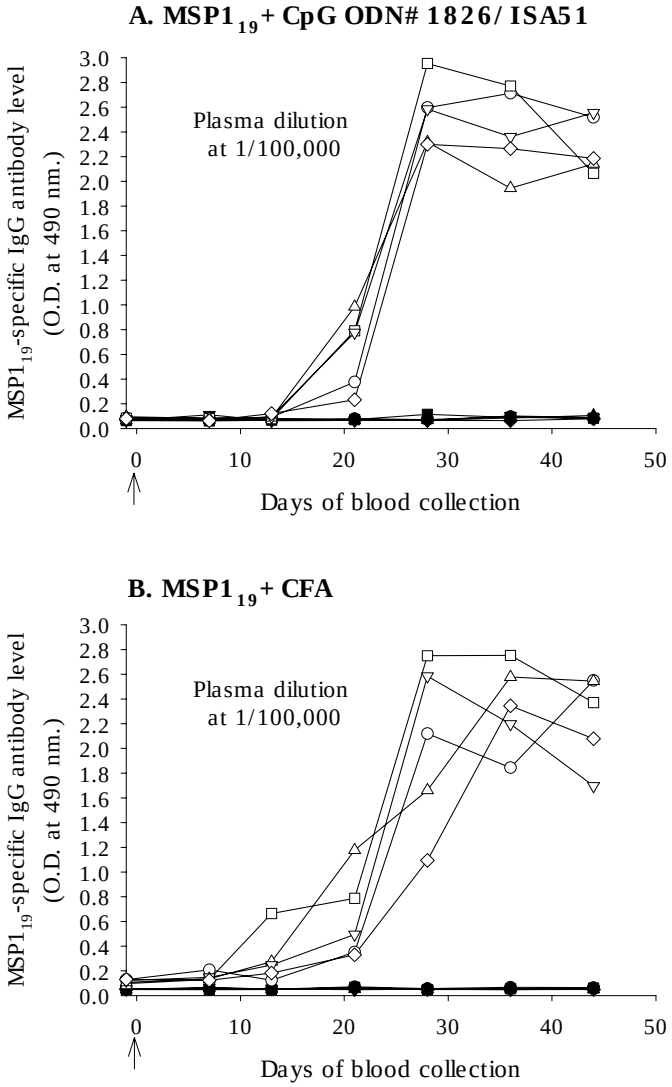

Figure 4 Kinetics of $MSP1_{19}$-specific antibody response in mice following a single-dose immunization with $MSP1_{19}$ formulated in CpG ODN#1826/Montanide ISA51or CFA. Five BALB/c mice were immunized with $MSP1_{19}$ (opened symbols) or PBS (closed symbols) mixed with CpG ODN#1826/Montanide ISA51 (A) or CFA (B) on day 0 via subcutaneous injection. Plasma was collected every week after immunization and assayed for $MSP1_{19}$-specific antibody by ELISA. Arrows indicate the day of immunization. One line represents one mouse.

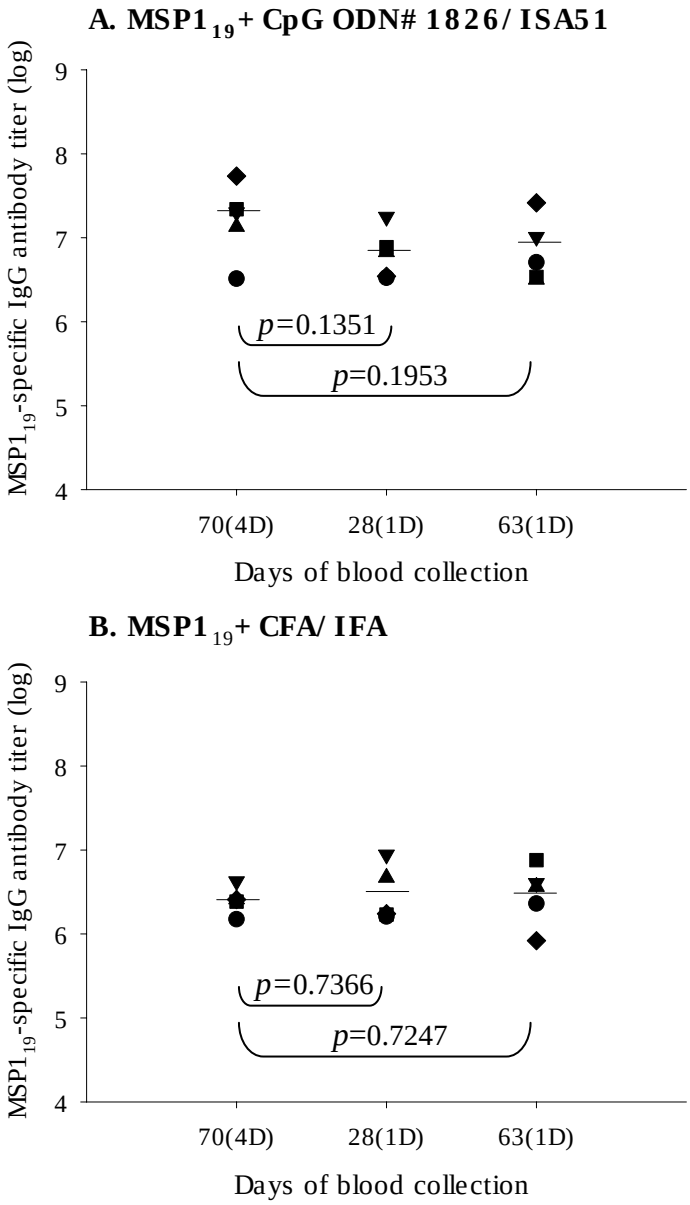

Figure 5 Comparison of $MSP1_{19}$-specific antibody levels induced between by single-dose and four-dose immunizations. Plasma of mice immunized with $MSP1_{19}$ mixed with CpG ODN#1826/Montanide ISA51 (A) or CFA/IFA (B) for four-dose collected at day 70 or for a single-dose collected at day 28 and 63 were assayed by ELISA for $MSP1_{19}$-specific antibody titer. 4D, four-dose immunization; 1D, single-dose immunization. One symbol represents one mouse.

(Figure 5A). Neither were the antibody titers of single-dose and four-dose immunized mice using CFA as adjuvant. The mean log antibody titer ± S.E. of single-dose immunization using CFA as adjuvant at day 28 and 63 post immunization were 6.459±0.333 and 6.465±0.357, and that of the four-dose immunization at day 70 post initial immunization was 6.401±0.160 (Figure 5B).

5. The Isotype of $MSP1_{19}$-Specific Antibody in Response to Immunization with $MSP1_{19}$ Mixed with CpG ODN#1826 and Montanide ISA51

The IgG1 and IgG2a isotype expression specific for $MSP1_{19}$ following single-dose and four-dose immunizations with $MSP1_{19}$ mixed with CpG ODN#1826/ Montanide ISA 51 or CFA/IFA were determined. The plasma of immunized mice was collected at day 70 related to the first immunization and assayed for the antibody titer. The results showed that in mice immunized either with a single or four doses of $MSP1_{19}$ the IgG1 antibody level was higher significantly than the IgG2a antibody level. In a single-dose immunization, the mean log IgG1 and IgG2a antibody titers ± S.E. were 6.601±0.229 and 3.956±0.259, respectively, $p<0.05$ using CFA and 7.143±0.208 and 5.981±0.134, respectively, $p<0.05$ using CpG ODN#1826/Montanide ISA51 (Figure 6B and C). In a four-dose immunization, the mean log IgG1 and IgG2a antibody titers ± S.E. were 6.692±0.177 and 4.407±0.427, respectively, $p<0.05$ using CFA/IFA, and 7.330±0.283 and 6.305±0.748, respectively, $p<0.05$ using CpG ODN#1826/Montanide ISA 51 (Figure 6B and C).

By comparison, $MSP1_{19}$-specific IgG1 antibody production in mice immunized with $MSP1_{19}$ mixed with CpG ODN#1826/Montanide ISA51 was significantly higher than that in mice immunized using CFA/IFA as adjuvant (mean log IgG1 antibody titer ± S.E., 7.330±0.283 *versus* 6.692±0.177, $p<0.01$, for the four-dose immunization; and 7.143±0.208 *versus* 6.601±0.229, $p<0.05$, for the single-dose immunization) (Figure 6B).

The $MSP1_{19}$-specific IgG2a antibody production in mice immunized with $MSP1_{19}$ mixed with CpG ODN#1826/Montanide ISA51 was significantly much higher than that in mice immunized using CFA/IFA as adjuvant (mean log IgG2a antibody titer ± S.E., 6.304±0.748 *versus* 4.407±0.427, $p<0.01$, for the four-dose immunization; and 5.981±0.134 *versus* 3.956±0.259, respectively, $p<0.000001$, for the single-dose

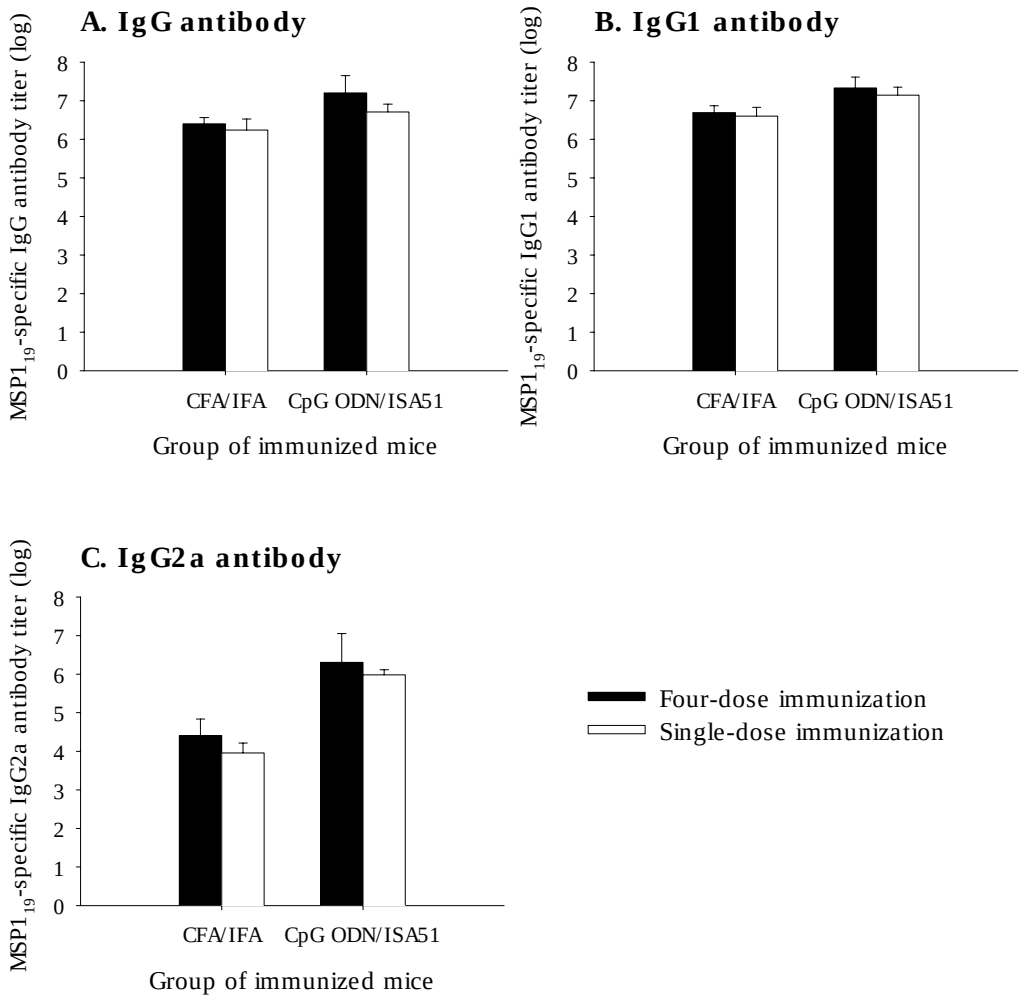

Figure 6 IgG1 and IgG2a antibody isotype specific for $MSP1_{19}$ produced in BALB/c mice after single-dose or four-dose immunization with $MSP1_{19}$ mixed with CpG ODN#1826/Montanide ISA51 or CFA/IFA adjuvants. Groups of five mice were immunized with a single dose or four doses of $MSP1_{19}$ mixed with CpG ODN# 1826/Montanide ISA51 or CFA/IFA as described in the Materials and Methods. Plasma was collected at day 70 and assayed for IgG (A), IgG1 (B), and IgG2a (C) antibody titer specific for $MSP1_{19}$ by ELISA.

immunization) (Figure 6C).

The ratio of $MSP1_{19}$-specific IgG1 and IgG2a antibody titer rising was compared between using CFA/IFA and CpG ODN#1826/Montanide ISA51 as adjuvants. Results showed that CpG ODN#1826/Montanide ISA51 enhanced the IgG1 antibody titer production with only 4.3- and 3.5-fold increase while it enhanced the IgG2a antibody production with 79.8- and 81.6-fold increase, compared to CFA/IFA in four-dose and single-dose immunization, respectively (Table 1).

Table 1 Ratio of $MSP1_{19}$-specific IgG1 and IgG2a antibody rising compared between CFA/IFA and CpG ODN#1826/Montanide ISA51 adjuvants used in $MSP1_{19}$-immunization.

Adjuvant used	**Geometric mean antibody titer**	
	IgG1	**IgG2a**
Four-dose immunization:		
CFA/IFA	4,926,517	25,541
CpG OND#1826/Montanide ISA51	21,374,698	2,016,043
Ratio of antibody titer rising (fold)	**4.3**	**79.8**
Single-dose immunization:		
CFA	3,986,575	9,039
CpG OND#1826/Montanide ISA51	13,903,288	737,700
Ratio of antibody titer rising (fold)	**3.5**	**81.6**

6. Duration of $MSP1_{19}$-Specific Antibody Response Induced by Immunization with $MSP1_{19}$ Mixed with CpG ODN#1826 and Montanide ISA51

After four-dose immunization with $MSP1_{19}$ mixed either with CpG ODN#1826/Montanide ISA51 or CFA/IFA, the $MSP1_{19}$-specific IgG antibody levels were monitored in the blood every month for up to 12 months since the last immunization. As shown in Figure 7, the antibody levels of mice adjuvanted with CpG ODN#1826/Montanide ISA51 did not decrease significantly over 12 months of study (i.e., the mean log antibody titers ± S.E. were 6.687±0.261 *versus* 6.904±0.147 at 1 and 12 months, respectively, after the last immunization, p=0.8478), while those of mice adjuvanted with CFA/IFA decreased gradually over the 12 months after the last immunization and showed a significant decrease when compared at 1 and 12 months (i.e., the mean log antibody titers ± S.E. were 6.465±0.118 *versus* 5.772±0.100, respectively, p<0.0001). Control mice immunized with PBS mixed with either adjuvant did not show any $MSP1_{19}$-specific antibody titer over the 12 months after the last immunization.

In addition, the longevity of the IgG1 and IgG2a antibody subclasses were evaluated by collecting sera at month 1, 6, and 12 and assayed by ELISA using the dilution of 1/100,000 and 1/1,000, respectively. Results showed that the IgG1 antibody levels in mice immunized four times with $MSP1_{19}$ plus CpG ODN#1826/Montanide ISA51 at month 6 (mean O.D. ± S.E.; 1.634±0.152) and 12 (0.969±0.130) postimmunization significantly lower than that at month 1 (2.659±0.131) (p=0.00093 and 0.00045, respectively). In the same manner, the IgG1 antibody levels in mice immunized four times with $MSP1_{19}$ plus CFA/IFA at month 6 (mean O.D. ± S.E.; 0.870±0.116) and 12 (0.501±0.212) postimmunization significantly lower than that at month 1 (1.552±0.084) (p=0.00143 and 0.00150, respectively) (Figure 8A). In contrast, the IgG2a antibody levels in mice immunized four times with $MSP1_{19}$ plus CpG ODN#1826/Montanide ISA51 or CFA/IFA were not significantly altered over the 12 months post immunization (p>0.05). The mean O.D. ± S.E. of $MSP1_{19}$-specific IgG1 antibody in mice immunized with $MSP1_{19}$ plus CpG ODN#1826/Montanide ISA51 were 1.317±0.066, 1.249±0.049, and 1.213±0.057 at 1, 6, and 12 months postimmunization, respectively, and in mice immunized with

$MSP1_{19}$ plus CFA/IFA were 0.687±0.060, 0.689±0.037, and 0.684±0.039 at 1, 6, and 12 months postimmunization, respectively (Figure 8B).

A question was asked further whether the antibody at 12 months after the last immunization was maximal or could be boosted. To address this, the immunized mice were given another dose of $MSP1_{19}$ mixed with CpG ODN#1826/Montanide ISA 51 or CFA/IFA. The results showed that the antibody titers did not increased significantly over 28 days after the booster using either adjuvants (i.e., the mean log antibody tiers ± S.E. were 6.997±0.122 [before boosting] *versus* 6.665±0.216 [28 days after boosting], p=0.172, for CpG ODN#1826/Montanide ISA 51 adjuvant; and 6.308±0.577 [before boosting] *versus* 6.066±0.487 [28 days after boosting], p=0.515, for CFA/IFA adjuvant) (Figure 9).

In addition, the duration of $MSP1_{19}$-specific IgG1 and IgG2a antibody responses after booster was also investigated and assayed by ELISA using the dilution of 1/100,000 and 1/1,000, respectively. Results showed that the IgG1 antibody levels in mice immunized four times with $MSP1_{19}$ formulated CpG ODN#1826/Montanide ISA51 or CFA/IFA increased significantly over 28 days after the booster (i.e., the mean O.D. ± S.E. were 0.937±0.048 [before boosting] *versus* 1.891±0.082 [28 days after boosting], p<0.0001, for CpG ODN#1826/Montanide ISA51 adjuvant; and 0.699±0.085 [before boosting] *versus* 1.631±0.174 [28 days after boosting], p<0.01, for CFA/IFA adjuvant) (Figure 10A). In contrast, the IgG2a antibody levels in mice immunized four times with formulated CpG ODN#1826/Montanide ISA51 or CFA/IFA were not significantly altered over 28 days after the booster (p>0.05). The mean O.D. ± S.E. of $MSP1_{19}$-specific IgG2a antibody in mice immunized with $MSP1_{19}$ formulated CpG ODN#1826/Montanide ISA51 were 1.216±0.084 and 1.082±0.079 at the day before boosting and 28 days after boosting, respectively, and in mice immunized with $MSP1_{19}$ formulated CFA/IFA were 0.865±0.085 and 0.754±0.132 at the day before boosting and 28 days after boosting (Figure 10B).

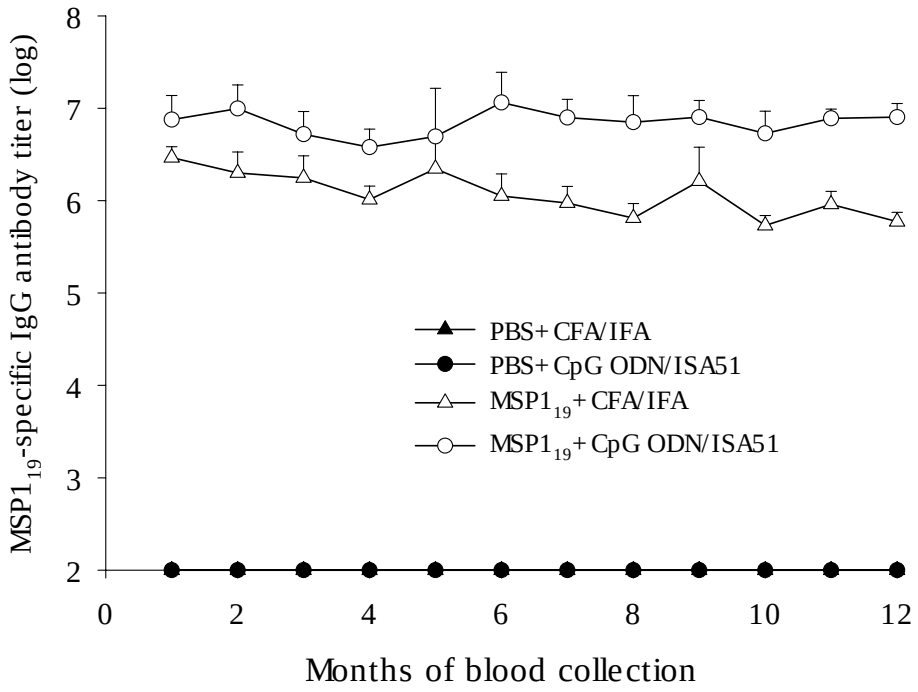

Figure 7 Duration of $MSP1_{19}$-specific IgG antibody response in mice immunized with $MSP1_{19}$ formulated with CpG ODN#1826/Montanide ISA51 and CFA/IFA. Groups of mice were immunized with $MSP1_{19}$ or PBS mixed with CpG ODN# 1826/Montanide ISA51 or CFA/IFA for four times as described in Materials and Methods. Plasma was collected every month over 12 months and assayed by ELISA for $MSP1_{19}$-specific IgG antibody titers.

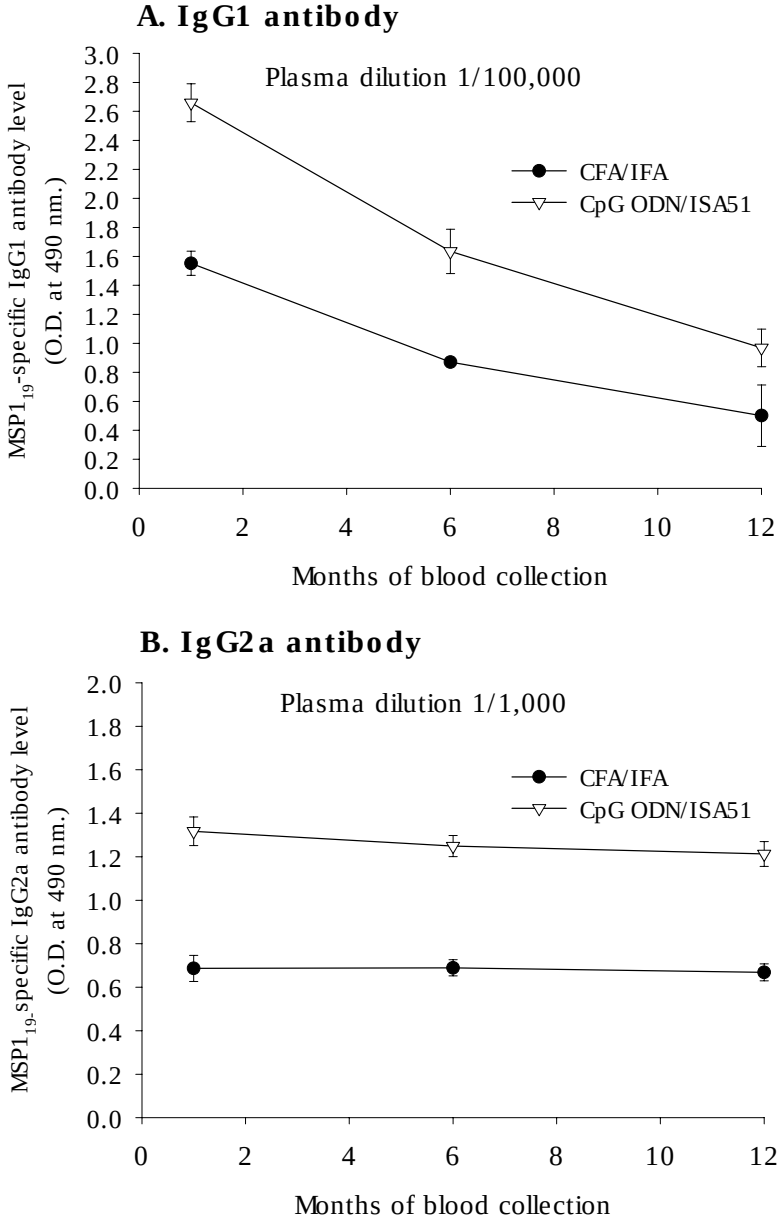

Figure 8 Duration of $MSP1_{19}$-specific IgG1 and IgG2a antibody responses in mice immunized with $MSP1_{19}$ formulated with CpG ODN#1826/Montanide ISA51 or CFA/IFA. Groups of mice were immunized with $MSP1_{19}$ mixed with CpG ODN# 1826/Montanide ISA51 or CFA/IFA for four times as described in Materials and Methods. Plasma was collected at month 1, 6 and 12 and assayed for $MSP1_{19}$-specific IgG1 (A) and IgG2a (B) antibody level by ELISA.

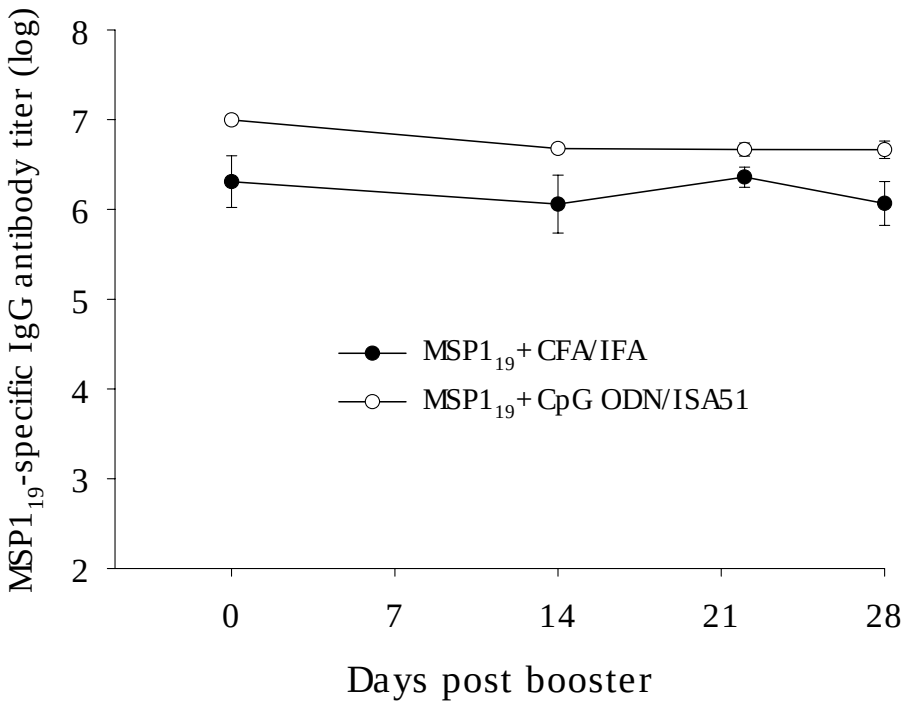

Figure 9 MSP1$_{19}$-specific antibody titers in 12 month-MSP1$_{19}$-immunized mice post booster.

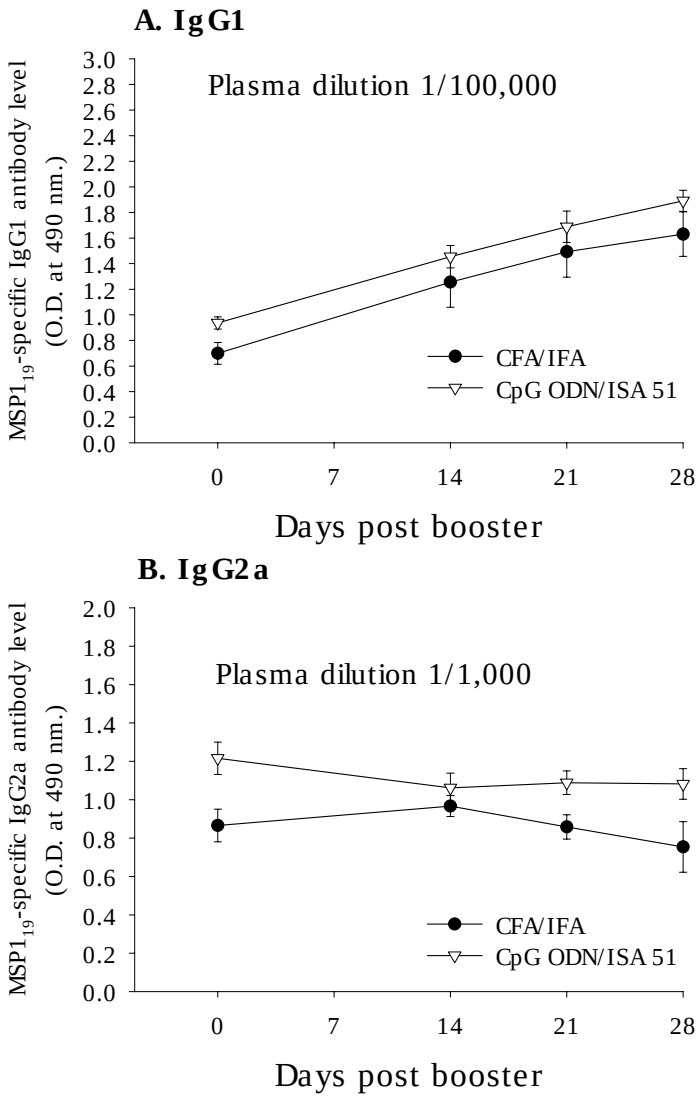

Figure 10 MSP1$_{19}$-specific IgG1 and IgG2a antibody levels in 12 month-MSP1$_{19}$-immunized mice post booster.

The duration of $MSP1_{19}$-specific IgG antibody response of mice immunized with a single-dose of $MSP1_{19}$ was also investigated for up to 22 weeks after immunization. After reaching the maximum level by 4-6 weeks after immunization with use of either adjuvants, the antibody levels were followed up for another 18 weeks (or week 22 since initial immunization). As can be seen in Figure 11, the antibody levels gradually decreased until week 22. The antibody levels at week 22 were significantly lower than those levels at week 4 i.e., mean O.D. ± S.E. at week 4 and week 22; 2.551±0.119 *versus* 1.870±0.104, $p<0.01$, with use of CpG ODN#1826/Montanide ISA51 (Figure 10A), and 2.042±0.304 *versus* 1.428±0.059 $p=0.0487$, with use of CFA in immunization (Figure 11). Control single-dose immunization with PBS with either adjuvant did not induce any $MSP1_{19}$-specific IgG antibody responses for up to 22 weeks of study.

Interestingly, the duration of $MSP1_{19}$-specific IgG1 and IgG2a antibody subclasses was evaluated by collecting plasma at week 6, 14, and 22 and assayed by ELISA using the dilution of 1/100,000 and 1/1,000, respectively. Results showed that the IgG1 antibody levels in mice immunized one time with $MSP1_{19}$ plus CpG ODN#1826/Montanide ISA51 rapidly decreased at week 14 (mean O.D. ± S.E.; 1.394±0.159) and 28 (0.746±0.098) compared to those at week 6 (2.024±0.236) with 31.2% and 62.4% lower levels, respectively. In the same manner, the IgG1 antibody levels in mice immunized with $MSP1_{19}$ plus CFA rapidly decreased at week 14 (mean O.D. ± S.E.; 0.831±0.077) and 28 (0.669±0.053) compared to those at week 6 (1.557±0.197) with 46.8% and 57.1% lower levels, respectively (Figure 12A). In contrast, the $MSP1_{19}$-specific IgG2a antibody levels in mice immunized one time with $MSP1_{19}$ plus CpG ODN#1826/Montanide ISA51 at week 14 (mean O.D. ± S.E.; 2.167±0.073) and 28 (2.113±0.0716) did not much change compared to those at week 6 (2.362±0.037) with only 8.5% and 10.6% changes, respectively. So did the IgG2a antibody levels in mice immunized with $MSP1_{19}$ plus CFA (mean O.D. ± S.E.; 0.698±0.036 at week 14; 0.636±0.034 at week 28; 0.620±0.024 at week 6) with 11.3% and 1.6% changes, respectively (Figure 12B).

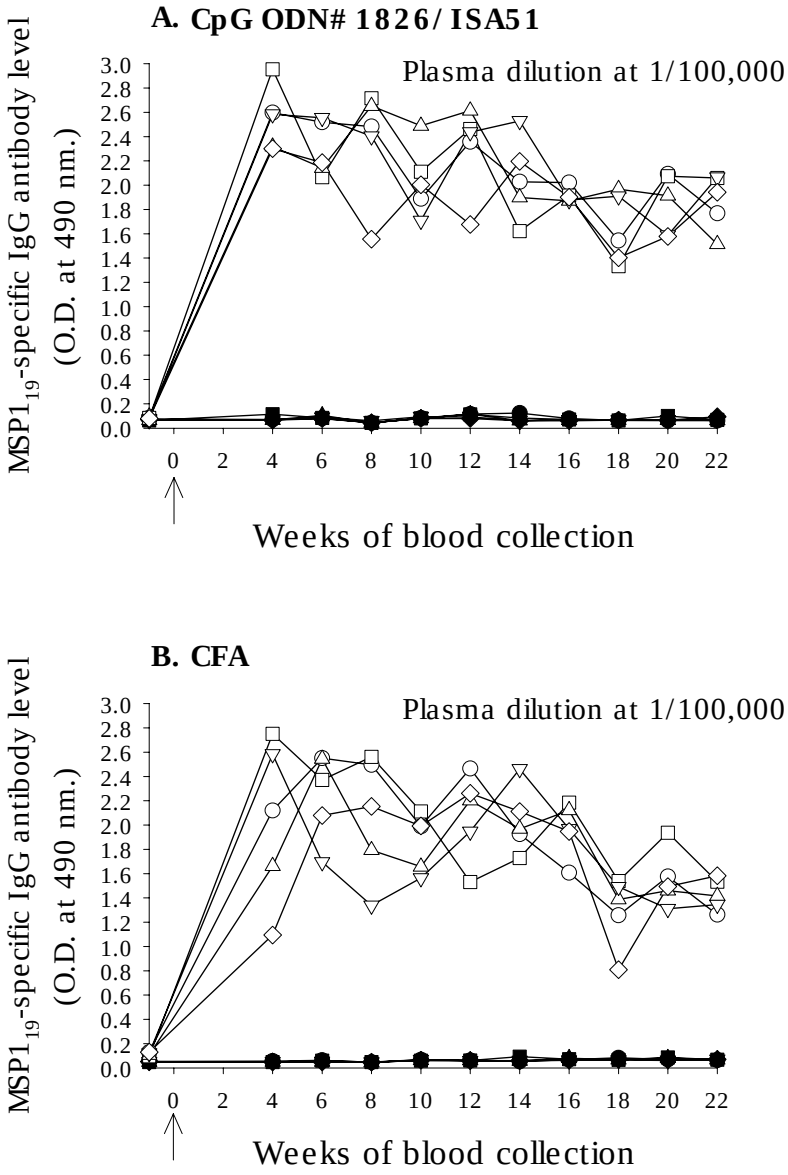

Figure 11 Duration of $MSP1_{19}$-specific antibody response in mice following a single immunization with $MSP1_{19}$ formulated CpG ODN#1826/Montanide ISA51 or CFA. Groups of five mice were immunized s.c. with $MSP1_{19}$ (opened symbols) or PBS (closed symbols) mixed with CpG ODN#1826/Montanide ISA51 (A) or CFA (B). Plasma was collected every two weeks after immunization and assayed by ELISA for $MSP1_{19}$-specific antibody. Arrows indicate the day of immunization. One line represents one mouse.

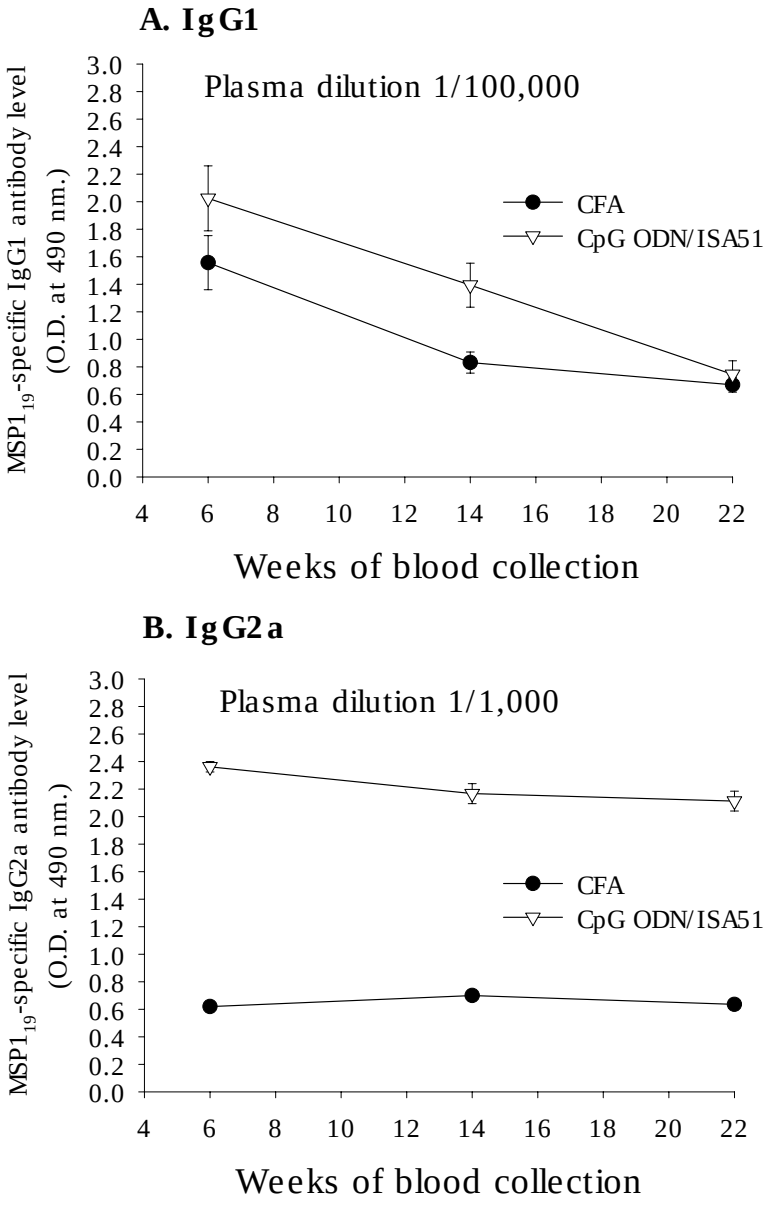

Figure 12 Duration of MSP1$_{19}$-specific IgG1 and IgG2a antibody response in mice following a single immunization with MSP1$_{19}$ formulated CpG ODN#1826/Montanide ISA51 or CFA.

7. Long Term Protective Response of Mice Immunized with $MSP1_{19}$ Against *P. yoelii* YM Infection

Having reported above that single-dose and four-dose immunization with $MSP1_{19}$ mixed with CpG ODN#1826 Montanide ISA51 or CFA/IFA induced high/comparable $MSP1_{19}$-specific IgG antibody with prolonged response, a question was asked whether the mice were still protective. To address this, all immunized mice were then challenged with $1x10^4$ live *P. yoelii*-pRBC. In four-dose immunization, the mice immunized with $MSP1_{19}$ plus CpG ODN#1826 in Montanide ISA51 were completely protected from the parasite infection with no parasitemia detectable over 36 days post challenge infection (Figure 13D). The mice immunized with $MSP1_{19}$ plus CFA/IFA were nearly completely protected; four of them survived infection with very low parasitemia during week 2 post challenge and one mice delayed infection for 10 days before parasiteimia rose up rapidly until the mouse died by day 18 (Figure 13C). Control mice immunized with PBS plus CpG ODN#1826 or CFA/IFA died with high parasiteimia within 8 days post infection except one mouse immunized with PBS plus CFA/IFA survived infection after it experienced high parasitemia and cleared the infection by day 18 (Figure 13A and B).

The single-dose immunized mice were also challenged with $1x10^4$ live *P. yoelii*-pRBC 22 weeks post immunization. As can be seen in Figure 12, the mice immunized with $MSP1_{19}$ plus CpG ODN#1826 in Montanide ISA51 or CFA were protected from *P. yoelii* infection all the mice survived infection with some experienced parasitemia. $MSP1_{19}$ plus CpG ODN#1826 in Montanide ISA51-immunized mice experienced lower parasitemia than CFA-immunized mice (Figure 14C and D). All control mice immunized with PBS plus CpG ODN#1826 in Montanide ISA51 or CFA died within 8 days with high parasitemia (Figure 14A and B).

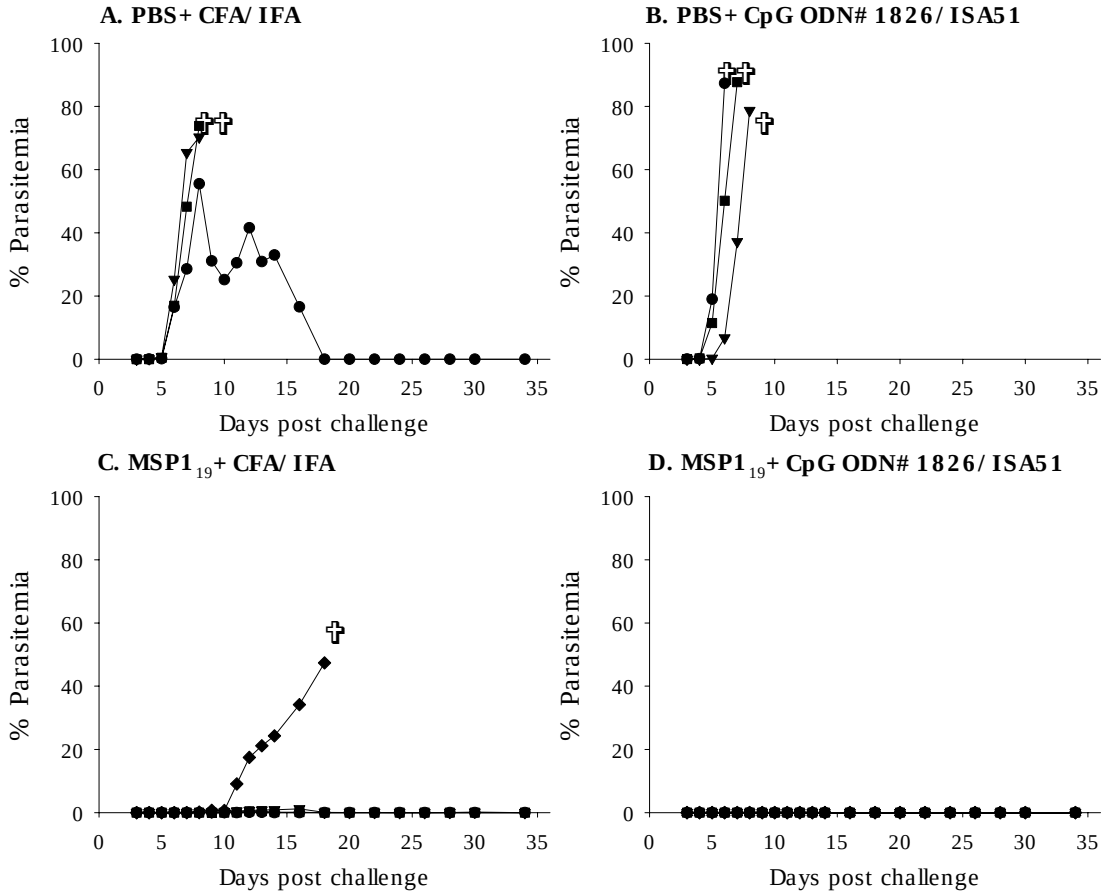

Figure 13 Protection of mice four-dose immunized with $MSP1_{19}$ for 12 months against *P.yoelii* YM infection. Mice were immunized with $MSP1_{19}$ or PBS plus CpG ODN# 1826/Montanide ISA51 or CFA/IFA for four-doses as described in Materials and Methods and left for 12 months before challenge infection with $1x10^4$ live *P.yoelii*-pRBC. Parasitemia was examined daily. The data show parasitemias of individual mice in each group. ✞, Dead.

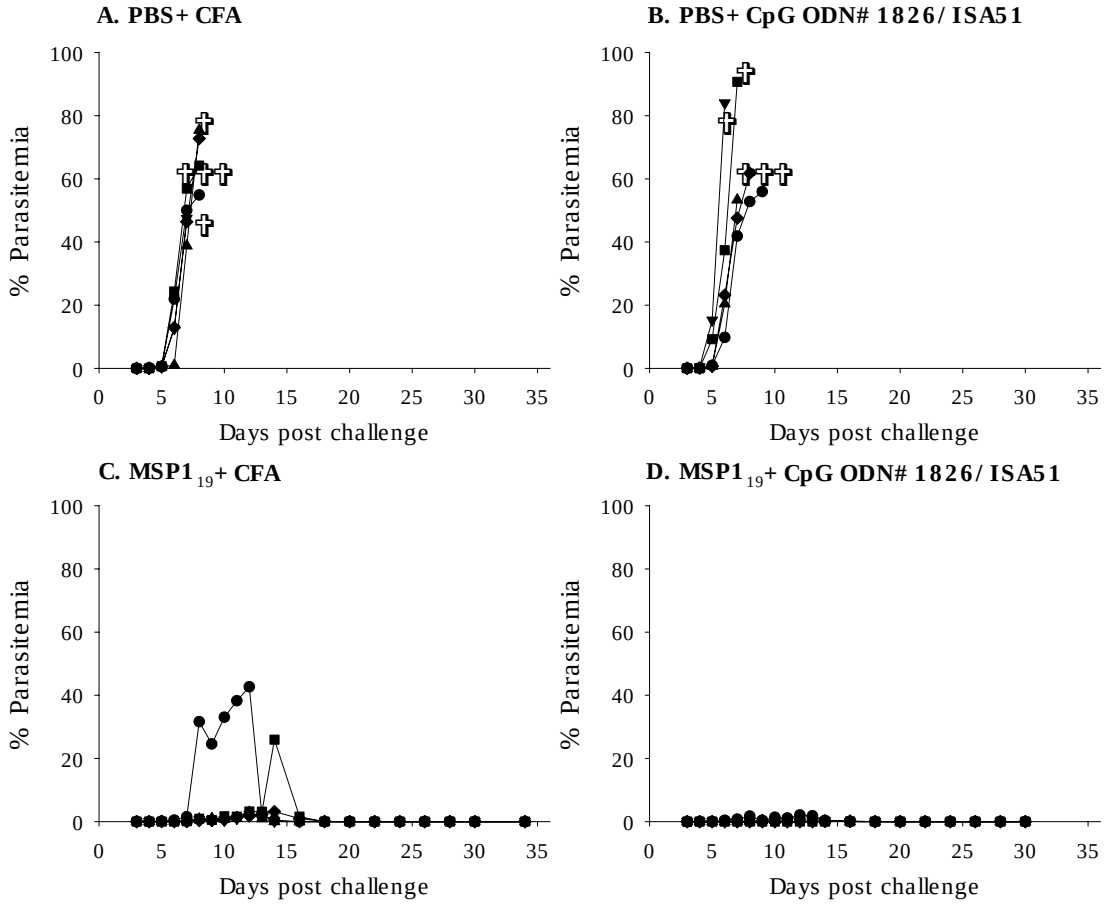

Figure 14 Protection of mice single-dose immunized with $MSP1_{19}$ for 22 weeks against *P.yoelii* YM infection. Mice were immunized with $MSP1_{19}$ or PBS plus CpG ODN# 1826/Montanide ISA51 or CFA as described in Materials and Methods and left for 22 weeks before challenge infection with $1x10^4$ live *P.yoelii*-pRBC. Parasitemia was examined daily. The data show parasitemias of individual mice in each group. ✞, Dead.

CHAPTER V
DISCUSSION

MSP1 is a leading malaria vaccine candidate, of which its conserved 42-and 19-kilodalton fragments can induce protective immunity against malaria parasite infection in mice (4, 118) and monkeys (119). These successful trials were based on the use of CFA/IFA as an adjuvant that is not allowed for human use because of its toxicity. The protective immunity is dependent mainly on the magnitude of antibody response and multiple immunization (4). The protection does not require the effector $CD4^+$ T cells as demonstrated in many evidences (4, 82). First, most mice immunized with $MSP1_{19}$ plus CFA/IFA were completely protected following *in vivo* depletion of $CD4^+$ T cells after immunization and just before parasite challenge. Second, B-cell knockout mice could not be immunized by $MSP1_{19}$. Third, mice adoptive transferred with $MSP1_{19}$-specific $CD4^+$ T cells were not protected against parasite infection. Forth, immunization of mice with defined $MSP1_{19}$ T cell epitopes were not protected after parasite infection. Several adjuvants have been tested to find out a proper one that can be use safely in human and effectively in induction of protective immunity. These included our previous studies that used a combination of adjuvant which is composed of CpG ODN#1826 and Montanide ISA51 or ISA720 (12). This study has demonstrated that the presence of CpG ODN#1826 in the vaccine formulation enhances the Th1 type immune response as evidenced by the huge increase of specific IgG2a antibody levels. No evidence has been shown whether the immunity induced by $MSP1_{19}$ immunization is long-lived. The present study claims to be the first time to show experimentally the longevity of $MSP1_{19}$-specific antibody response following immunization. Mice immunized with the recombinant *P. yoelii* $MSP1_{19}$ in CpG ODN#1826/Montanide ISA51 using a standard immunization protocol (four immunizations) induced $MSP1_{19}$-specific IgG antibody response which persisted at high level for at least 12 months.

Montanide ISA51 was used in this study because it is more potent in induction of $MSP1_{19}$-specific antibody response and protection against blood-stage *P. yoelii* challenge infection, than Montanide ISA720. The effect of both adjuvants on the responses of IgG antibody subclasses are different, in that Montanide ISA51 gives rise to more IgG1 antibody response than Montanide ISA720, but both of them are equal in induction of IgG2a antibody response (12). When CpG ODN#1826 was included in both Montanide adjuvants, both IgG1 and IgG2a antibody responses were enhanced and the responses were higher than the use of CFA/IFA as adjuvant. However, IgG2a antibody response was more enhanced than IgG1 antibody response. These findings have been confirmed again in this study (Table 1). Both Montanide ISA51 and 720 adjuvants have been clinical tested and are safe for human use (120, 121).

Other than conferring protection against blood-stage malaria infection, $MSP1_{19}$ immunization can induce protection against exoerythrocytic stage malaria infection. Recent study by Kumar et al (112) demonstrated that mice immunized with $MSP1_{19}$ mixed with Montanide ISA51 plus CpG ODN#1826 were protected against *P. yoelii* sporozoite challenge infection. The protection was mediated by antibody-mediated immunity but not by T cell-mediated immunity since when the immunized mice were depleted *in vivo* of interleukin-12 and IFN-γ cytokines and $CD4^+$ and $CD8^+$ T cells, no significant effect on immunity against sporozoite challenge was observed (112).

In this study we have shown that immunization of mice with $MSP1_{19}$ formulated CpG ODN/Montanide ISA51 or CFA/IFA is able to induce long-termed memory. The persistent of memory may involve many factors. The first factor is the slowly continuous stimulation of the immune system by the vaccine formulation, in which Montanide ISA51, an oil-based adjuvant can be emulsified to incorporate the vaccine as well as CpG ODN into the emulsion that releases both of them slowly to stimulate the immune system continuously. Immunization with $MSP1_{19}$ alone or together with CpG ODN has been shown not to be able to induce protective immunity (12). The second factor is the booster effect or repeating immunizations. As evidenced in this study, the results demonstrated that while the IgG antibody response to $MSP1_{19}$ induced by multiple immunization could persist at high level for at least 12 months with no significant decrease (Figure 7), the IgG antibody response induced by single immunization could not (Figure 10). In addition, other factors of the host

immune response and the nature of antigen may involve in the longevity of the antibody response. These include the persistence of the antigen on the follicular dendritic cells, the generation of long-lived plasma cells, or reduced levels of plasma cells apoptosis (122). Moreover, T cells play a major role in controlling the survival of antigen-specific B cells (plasma cells and memory B cells). It has been shown that T cells rescue B cells from apoptosis through the interaction of CD40/CD40L during the initial phase of the immune response (123), followed by driving the multiplication of B cells in the germinal centers and the isotype switching.

Unmethylated CpG dinucleotide sequences present in bacterial genomes or as a synthetic CpG ODN plays a role in stimulation of immune response in mammals to a vaccine by a variety of mechanisms, particularly in the arm of Th1 type immune response. CpG DNA also involves in B cell-mediated immune responses. Recent studies have shown that CpG DNA induces the B cell response by enhancing terminal differentiation (124), induces B cell proliferation (125), interferes the programmed cell death or apoptosis of B cells (122). With the low dose of CpG ODN promote antigen-specific immune responses by synergistically acting in concert with B-cell antigen receptors (9). CpG ODNs can also stimulate monocyte, macrophage, and dendritic cells to express increased levels of costimulatory molecules and to secrete high levels of certain cytokines such as IFN-γ and IL-12 (126). These cytokines enhance NK cell activity in IFN-γ synthesis and lysis of target cells (113). CpG ODNs also stimulate monocytes to differentiate to mature and functional dendritic cells that is capable of supporting the humoral and cell-mediated immune responses (127). These properties were supported later by many studies. They demonstrated that the injection of CpG DNA alone rendered protection against a variety of allergens and infectious agents by non-antigen-dependent mechanisms (128-130) and promoted protective antigen-specific immunity (12, 131-133). Here in this study, mice immunized with $MSP1_{19}$ in Montanide plus CpG ODN#1826 produced specific IgG2a antibody response higher many folds than mice immunized $MSP1_{19}$ in CFA/IFA (Table 1) or in Montanide alone (12). Addition of CpG ODN in $MSP1_{19}$ formulated with alum also showed the enhancement of specific IgG antibody production and the induction of partial protection against *P. y*oelii infection in mice (133).

Even though the $MSP1_{19}$-specific IgG antibody response induced by immunization using CpG ODN#1826/Montanide ISA51 as adjuvant could persist long-lived, studies in the subclass of IgG1 and IgG2a antibody responses showed different longevity. The $MSP1_{19}$-specific IgG1 antibody levels decreased significantly 6 months after the last immunization either using CpG ODN#1826/Montanide ISA51 or CFA/IFA as adjuvant but the IgG2a antibody levels were not altered significantly over the 12 months of observation after the last immunization (Figure 8). It has been known that IgG1 antibody response represents Th2 immune response and IgG2a antibody response represents Th1 immune response. The different longevity of antibody subclass responses, therefore, reflects the bias of the memory responses of Th1- and Th2-type immunity. Thus, our findings suggest that the lifespan of Th2 memory to $MSP1_{19}$ is shorter than that of the Th1 one. The mechanism of failure in maintenance of the $MSP1_{19}$-specific Th2 memory is not known and needed to be studied further.

The previous study reported by Hirunpetcharat et al. (4) has demonstrated that the number of immunization influences on the outcome of the antibody level and protective activity against malaria parasite infection. Immunization with four-doses (at day 0, 21, 42, 56) of $MSP1_{19}$ in CFA/IFA was minimum to induce complete protective immunity; three-dose showed some patent parasitemia (4). Surprisingly, in this study, a single-dose immunization is capable of inducing antibody response to $MSP1_{19}$ in the similar level to the four-dose immunization. This can be seen when the $MSP1_{19}$-specific IgG antibody level of four-dose immunized sera collected at day 70 since the first immunization was compared to the antibody levels of single-dose-immunized sera collected at day 28 and 63 (Figure 5). The kinetics of the antibody response by a single-dose immunization showed that the antibody level increased rapidly after day 14 and reached the maximum level by day 28 (Figure 4), suggesting that the vaccine formulation continuously stimulated the immune response. However, the capable of generating the memory response to $MSP1_{19}$ by a single-dose immunization was less than that by the four-dose immunization. This may be because memory response needs a booster. The continuous stimulation of the immune response by a single immunization may not be enough to induce the strong memory response, even though it is able to induce the magnitude of the antibody response.

Another reason may be that a booster dose is given a short interval that leads to cause death of effector B cells by apoptosis (122).

In conclusion, our findings have shown that multiple immunization with $MSP1_{19}$ formulated with CpG ODN in Montanide ISA51 is more potent in induction of the specific IgG antibody response and in persistence of antibody response than the immunization formulated with CFA/IFA. A single immunization induces the comparable magnitude of antibody response but less in persistence of antibody response. Nevertheless it may be useful in application to individuals in malarial endemic areas where natural infection would induce the booster effect and consequently result in long-term protective immunity.

CHAPTER VI
CONCLUSION AND RECOMMENDATION

This study aimed to investigate the duration of $MSP1_{19}$-specific antibody response following immunization with the recombinant *P. yoelii* $MSP1_{19}$ in two adjuvant formulations, CpG ODN#1826/Montanide ISA51 and CFA/IFA. Four-dose and single-dose immunizations were also studied. Following the four-dose immunization, $MSP1_{19}$-specific IgG antibody levels were higher in mice immunized with $MSP1_{19}$ mixed with CpG ODN#1826/Montanide ISA51 than in mice immunized with $MSP1_{19}$ mixed with CFA/IFA. The longevity of $MSP1_{19}$-specific IgG antibody response lasted longer than 12 months either by immunization with CpG ODN#1826/Montanide ISA51 or CFA/IFA formulation but the antibody response of the former formulation was more persistent. In contrast, the $MSP1_{19}$-specific IgG antibody level following the single dose immunization with $MSP1_{19}$ mixed with CpG ODN#1826/Montanide ISA51 or CFA gradually decreased to a significantly lower level within 22 weeks, even though the antibody response was raised to the maximum level by 4 weeks after immunization. The antibody levels were comparable to that of the four-dose immunization.

The $MSP1_{19}$-specific antibody subclass responses were investigated, particularly IgG1 and IgG2a, based on the Th1- and Th2-armed immune responses, respectively. Following the four-dose immunization with $MSP1_{19}$ formulated either with CpG ODN#1826/Montanide ISA51 or CFA, the $MSP1_{19}$-specific IgG1 antibody level was higher than the IgG2a antibody level. Both IgG1 and IgG2a antibody titers were higher in $MSP1_{19}$-immunization formulated with CpG ODN#1826/Montanide ISA51 than that formulated with CFA/IFA. Comparison to immunization with $MSP1_{19}$/CFA/IFA formulation, immunization with $MSP1_{19}$/CpG ODN#1826/ Montanide ISA51 formulation promoted more increase of IgG2a antibody titer than that of IgG1 antibody titer (79.8-fold and 4.3-fold by four-dose immunization, and 81.6-fold and 3.5-fold by single-dose immunization). These findings suggest that CpG

ODN#1826/Montanide ISA51 enhance Th1-type immune response. Over the 12 months of follow up, the IgG1 antibody titer decreased significantly but not the IgG2a antibody titer, suggesting that $MSP1_{19}$-specific IgG2a antibody persisted lasts longer than $MSP1_{19}$-specific IgG1 antibody.

When the mice that had been immunized with $MSP1_{19}$ formulated either in CpG ODN#1826/Montanide ISA51 or in CFA/IFA for 12 months were challenged with a lethal *P. yoelii*, they were protected against the infection with the former group having lower patent parasitemia than the latter group. This indicates that the ability of protection corresponded to the level of $MSP1_{19}$-specific IgG antibody.

Taken together, these findings lead to an application to human malaria vaccine development, particularly on the vaccine design in induction of the strong protective and long-lasting immune response. By this study, even though a single-dose immunization provided shorter-term of immune response, the antibody level was as high as four-dose immunization. These lead to hypotheses whether the application of a single-dose immunization in human would be as effective as in mice and whether a natural infection of a single-dose immunized individual would maintain the protective antibody response. These questions need to be proved further.

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