Formalin-killed Blood-stage *Plasmodium yoelii* Ajuvanted with CpG Oligodeoxynucleotide and Montanide Induces Protective Immune Responses Against Malaria Infection in Mice

Plasmodium yoelii เชื้อตายด้วยฟอร์มาลินร่วมกับแอดจูแวนท์ CpG Oligodeoxyneucleotide และ Montanide ชักนำให้เกิดการตอบสนองของภูมิคุ้มกันต่อการป้องกันการติดเชื้อมาลาเรียในหนูทดลอง

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ABSTRACT

This study aimed to investigate the immune response of BALB/c mice after immunization with formalin-killed blood stage *P. yoelii* formulated with Montanide ISA720 plus CpG ODN. The results showed that immunization with $3x10^7$ formalin-killed *P. yoelii*-pRBC formulated with the adjuvants induced *P. yoelii*-specific antibody responses and protected mice against live *P. yoelii* challenge infection. The lower numbers of formalin-killed *P. yoelii*-pRBC with those adjuvants were not able to induce any antibody response and protection against challenge infection either. The determination of *P. yoelii*-specific antibody isotypes of the immune mice showed that IgG1 antibody titers were highest, followed by IgG2a, IgG2b and IgG3.

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อตรวจสอบการตอบสนองของภูมิคุ้มกันหลังจากการกระตุ้นภูมิคุ้มกันด้วย *P. yoelii* ที่ฆ่าด้วยฟอร์มาลินร่วมกับ Montanide ISA720 และ CpG ODN ผลการศึกษาพบว่าระดับของ *P. yoelii*-specific antibody ในหนูกลุ่มที่ได้รับ *P. yoelii* เชื้อตายปริมาณ 3x107pRBC ร่วมกับแอดจูแวนท์ดังกล่าว สามารถชักนำให้เกิดการตอบสนองของ antibody ที่จำเพาะต่อเชื้อ P. yoelii และสามารถป้องกันการติดเชื้อภายหลังให้การติดเชื้อด้วย *P. yoelii* เชื้อเป็น หนูกลุ่มที่ได้รับ *P. yoelii* เชื้อตายในปริมาณที่ต่ำกว่าร่วมกับแอดจูแวนท์นั้น ไม่สามารถชักนำให้เกิดการตอบสนองของ antibody และไม่สามารถป้องกันการติดเชื้อได้ การตรวจหาชนิดของ antibody ที่จำเพาะต่อเชื้อ *P. yoelii* พบว่า ระดับของ IgG1 มีสูงสุด รองลงมาคือ IgG2a, IgG2b และ IgG3 ตามลำดับ

Key Words : *Plasmodium yoelii*, CpG ODN, Immunization
คำสำคัญ : พลาสโมเดียมโยลิอาย ซีพีจี โอดีเอ็น การกระตุ้นภูมิคุ้มกัน

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Introduction

Malaria is one of leading public health problems, causing morbidity of approximately 515 million and mortality of 1-3 million world populations with the majority of deaths being in young children in sub-Saharan Africa (Snow *et al.*, 2005). Malaria is caused by four species of the genus *Plasmodium*, and the most severity of the disease is caused by *P. falciparum*. Although efforts of malaria prevention have shifted toward more appropriate local protection methods, such as partial control of breeding grounds and use of insecticide-treated mosquito net, the malarial control measures remain difficult because of the emergence of insecticide-resistant mosquito vectors and drug-resistant parasites (Aikins *et al.*, 1998; Utzinger *et al.*, 2001). Moreover, the ecological control has been at risk because of global warming and population movement. Therefore, a vaccine for malaria is urgently needed.

Many malaria vaccine candidates including recombinant proteins and DNA formula have been clinically tested but none is close to licensure. Recently, a small clinical trial of vaccination with ultra low dose of live *P. falciparum* has been shown successfully to induce protective immunity, of which PBMCs proliferate and secrete IFN-γ, but not IL-4 and IL-10 in response to parasite stimulation (Pombo *et al.*, 2002). Similarly, sub-patent infection with *P. chabaudi* can induce immunity of mice against challenge infection with a large dose of both homologous and heterologous parasites and induces higher IFN-γ^+ than IL-4^+ producing $CD4^+$ lymphocytes, suggesting a predominant Th1-type response (Elliott *et al.*, 2005). However, mass preparation of such live vaccine is not practical for public use. Therefore, this study will focus on an experimental trial of immunization with formalin-killed *P. yoelii* formulated with CpG oligonucleotide (ODN) and Montanide, which are highly effective adjuvants (Hirunpetcharat *et al.*, 2003).

Materials and Methods

Study design

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

Mice and parasites

Female BALB/c mice, 6-8 weeks of age purchased from The National Laboratory Animal Center, Mahidol University, Salaya, Nakhon Pathom, Thailand, were used in all experiments. The animal ethics was approved by the Faculty of Tropical Medicine-Animal Care and Use Committee, Faculty of Tropical Medicine, Mahidol University. *P. yoelii* YM, a lethal murine malaria parasite, was maintained in our laboratory and used for antigen preparation and challenge infection.

Adjuvants

CpG ODN1826 (TCCATGACGTTCCTGACGTT) kindly provided by Prof. Arthur Krieg, Coley Pharmaceutical Group, and Montanide ISA720 (Seppic, France) were used in this study.

Preparation of formalin-killed *P. yoelii*

Blood was collected from mice infected with *P. yoelii* YM when parasitemia was up to 40-50%. Blood was diluted with PBS and centrifuged at 1,500 rpm for 10 min. Buffy coat was discarded and red blood cells were washed twice with PBS. After the last wash, supernatant was discarded and packed cells were added with 5 packed cell volumes (PCV) of 0.1% formalin in PBS. The mixture was left overnight at 4°C. The lysis of red blood cells was seen. The parasites were washed at least five times with PBS and resuspended to

$1.5x10^9$ *P. yoelii*-pRBC (relative to the original concentration unit).

Immunization of mice with formalin-killed *P. yoelii*

Groups of eight mice were immunized with 100 µl of 30, $3x10^3$, $3x10^5$, or $3x10^7$ formalin killed *P. yoelii* mixed with 50 µg CpG ODN1826 and Montanide ISA720 via s.c., s.c., i.p., and i.p. injections on day 0, 21, 42 and 56, respectively (Hirunpetcharat *et al.*, 1997). A control group was immunized with PBS instead of the killed *P. yoelii*.

Plasma collection

Twenty microliters of blood was collected from mouse-tail vein and 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 10 min. Plasma was collected into a new tube and stored at -20°C until assayed.

Preparation of crude *P. yoelii* soluble antigen

Blood was collected from mice infected with *P. yoelii* YM when parasitemia was up to 40-50% by cardiac puncture into a heparinized tube and washed three times with PBS. After the last wash, supernatant was discarded and the erythrocyte pellet was resuspended in 0.01% saponin in PBS (1 ml per 0.2 ml PCV) and incubated for 20 min at 37°C with frequent shaking. Then cold PBS was added and the tube was centrifuged at 1,500 rpm for 10 min at 4°C. The supernatant was discarded. The pellet was washed twice with cold PBS and resuspended with PBS. The parasite suspension was sonicated at 4°C five times with 1 min blasts, and centrifuged at 10,000 rpm for 10 min. Supernatant was collected and dialyzed twice against one liter of PBS each time overnight at 4°C. The concentration of crude parasite antigen was determined using BCA assay kit (Thermo Fisher Scientific, Inc., Rockford, USA). The antigen was kept into aliquots at -20°C until use.

Antibody assay by ELISA

ELISA for antibody assay was conducted as described by Hirunpetcharat *et al*, (2003). Briefly MaxiSorp plates (Nalge Nunc International, Rochester, NY) were coated with 100 µl/well of 10 µg/ml of crude *P. yoelii*-soluble antigen in coating buffer, and incubated overnight at 4°C. After three washes with 0.05% Tween/PBS, wells were blocked with 200 µl of 3% skim milk/PBS at 37°C for 1 h. Supernatant was discarded, and 100 µl of two-fold serial dilutions of plasma samples were added. After incubation for 1 h, wells were washed and then 100 µl of 1/3,000 diluted horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG (Zymed Laboratory, Inc., San Fransisco, CA) was added. For determining the IgG antibody isotype, after wells were washed, 100 µl of 1/1,000 diluted HRP-conjugated anti-mouse IgG1, IgG2a, IgG2b or IgG3 antibody (Zymed Laboratory, Inc., San Fransisco, CA) was added. After incubation for 1 h, wells were washed, and *o*-phenylenediamine (OPD; Sigma Chemical Company, St Louis, MO, USA) substrate solution was added. After incubation for 30 min at room temperature, 1N H_2SO_4 was added and optical density (OD) was read at a wavelength of 490 nm by ELISA reader. Antibody titer was judged as the highest dilution of immunized plasma that gives OD equal to or greater than the mean OD of normal unimmunized plasma control.

Challenge of mice with *P. yoelii*

Mice were infected i.v. with $1x10^4$ live *P. yoelii* YM-pRBC. The parasitemia was monitored daily by microscopic examination of Dip-Quick-stained blood smears.

Statistical analysis

The statistical difference of OD or titer between the experiment and control groups was analyzed using Students' *t* test. A P-value of 0.05 or less was considered statistical significance.

Results and discussion

Antibody response after immunization with formalin-killed *P. yoelii*-pRBC mixed with Montanide ISA720 and CpG ODN.

To evaluate antibody response post immunization, groups of 3 or 4 mice were immunized with 100 μl of a vaccine regimen containing PBS or 30, $3x10^3$, $3x10^5$ or $3x10^7$ formalin-killed *P. yoelii*-pRBC formulated with Montanide ISA720 and CpG ODN 1826 via s.c., s.c., i.p., and i.p. injections on day 0, 21, 42 and 56, respectively. Plasma was collected 13 days after the last immunization and assayed for antibody level by ELISA.

As show in Table 1, *P. yoelii*-specific IgG antibody titers of mice immunized with PBS or 30, $3x10^3$ and $3x10^5$ formalin killed *P. yoelii*-pRBC mixed with the adjuvants were ≤100 but the mice immunized with $3x10^7$ formalin-killed *P. yoelii*-pRBC formulated with the adjuvants produced high antibody titers of 819,200 of all three mice.

This suggests that the acquisition of specific antibody responses by killed *P. yoelii* immunization is dose-dependent and, in this case, the minimum amount of at least $3x10^7$ pRBC is required.

Kinetics of P. yoelii-specific antibody response following immunization with formalin-killed *P. yoelii*-pRBC mixed with Montanide ISA720 and CpG ODN.

We then evaluated the kinetic of *P. yoelii*-specific antibody response by collecting blood one day just before each immunization as well as about two weeks after the last immunization, and assayed for the antibody level by ELISA. As can be seen in Figure 1, the antibody level of mice immunized with $3x10^7$ formalin-killed *P. yoelii*-pRBC mixed with Montanide ISA720 plus CpG ODN could be detected with modest increase after the second immunization (mean OD ± SE at plasma dilution of 1/800; 0.671±0.102), then increased more rapidly, and reached to the peak after the third immunization (mean OD ± SE; 1.488±0.271). After the fourth immunization, the antibody level did not decrease (mean OD ± SE; 1.295±0.277) any more. The antibody levels of mice immunized with lower number of the killed *P. yoelii* with the adjuvants could not be detected at any time points during 70 days of immunization. This suggests that the maximum antibody response requires boosting immunization, and the optimum dose of antigen.

Table 1 Antibody titers after immunization with PBS or formalin killed *P.yoelii*-pRBC formulated with Montanide ISA720 and CpG ODN at day 69; day just before challenge infection.

Group of mice	Number of mice	Geometric mean of *P. yoelii*-specific antibody
A: PBS + Montanide + CpG-ODN	4	≤100
B: 30 pRBC + Montanide + CpG-ODN	4	≤100
C: $3x10^3$ pRBC + Montanide + CpG-ODN	4	≤100
D: $3x10^5$ pRBC + Montanide + CpG-ODN	4	≤100
E: $3x10^7$ pRBC + Montanide + CpG-ODN	3	819,200

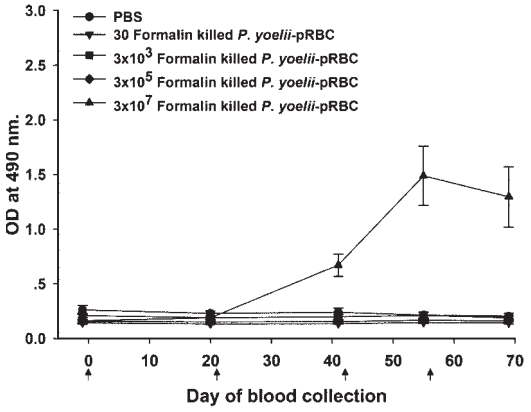

Figure 1 Kinetics of antibody response of BALB/c mice to *P. yoelii* crude antigen. Groups mice were immunized with PBS or different amounts of formalin-killed *P. yoelii*-pRBC formulated with CpG ODN and Montanide ISA720 on day 0, 21, 42 and 56 via s.c., s.c., i.p., and i.p. routes, respectively. Plasma collected at the indicated time points was diluted to 1/800 and determined for *P. yoelii*-specific IgG antibody by ELISA. Data show mean OD ± SE at 490 nm. ↑ indicates the time point of immunization.

Isotype of *P. yoelii*-specific antibody

Having shown that only the group of mice immunized with $3x10^7$ formalin killed *P. yoelii*-pRBC with the adjuvants produced the specific IgG antibody, we then evaluated the isotype response. It was found that the IgG1 antibody titers were highest followed by IgG2a, IgG2b and IgG3 with the mean log antibody titers ± SE being 6.034± 0.074, 4.468±0.292, 3.505±0.190, and ≤2.000, respectively (Figure 2). These findings suggest that the Th1 and Th2-type responses are elicited. However, the Th1- response enhancing effect of CpG ODN is not cleared yet in this experiment.

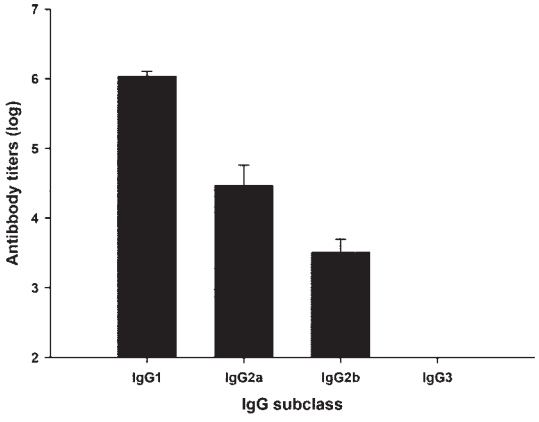

Figure 2 Isotype of specific antibodies. Mice were immunized with formalin-killed P. yoelii 3x107 pRBC formulated with Montanide ISA720 and CpG ODN as described in materials and methods. Plasma was collected at day 69 or day just before challenged, two-fold diluted, and assayed for the antibody isotype by ELISA. Data show geometric mean antibody titers and SE (error bars).

Protection against challenge with live *P. yoelii* - pRBC

Thirteen day after the last immunization, all groups of mice were challenge i.v. with $1x10^4$ live *P. yoelii* YM-pRBC. It was found that mice immunized with PBS (a control group) or 30, $3x10^3$ and $3x10^5$

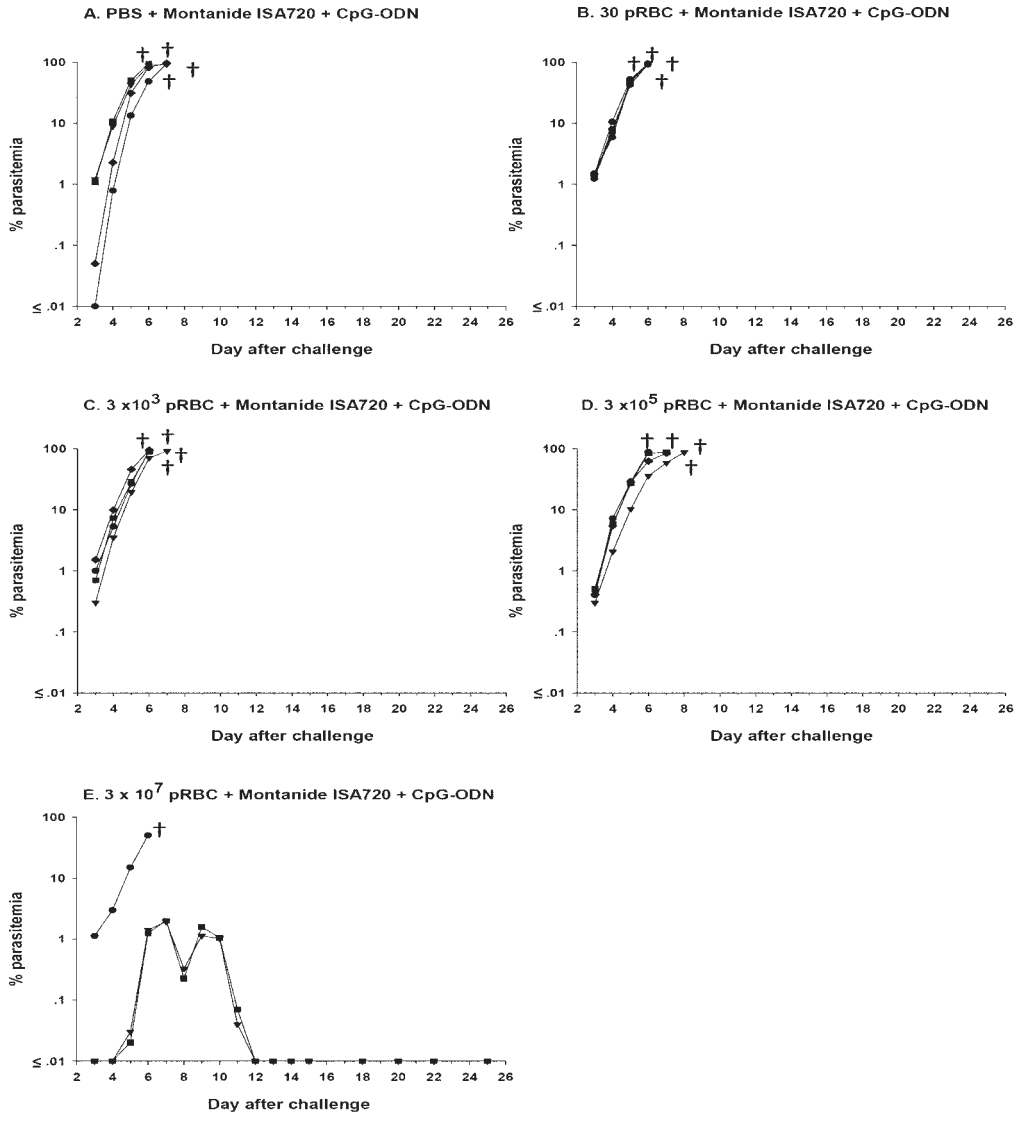

Figure 3 Parasitemia of immunized mice after challenge infection. Mice were immunized with PBS or formalin-killed *P. yoelii*-pRBC formulated with CpG ODN and Montanide ISA 720 at day 0, 21, 42 and 56 via s.c., s.c., i.p., and i.p. injections, respectively. Thirteen days later, mice were challenged with 1×10^4 live *P. yoelii*-pRBC and parasitemia was monitored daily. Data show parasitemia of individual mice in each group. †, death formalin-killed *P. yoelii*-pRBC mixed with Montanide ISA 720 and CpG ODN were not protected and they died with high parasitemia within 8 days after infection (Figure 3A, B, C and D). Only mice immunized with $3x10^7$ formalin killed *P. yoelii*-pRBC mixed with the adjuvants could be protected after infection (Figure 3E). Two of the three immunized mice survived with experiencing less than 3% parasitemia before being cleared on day 12 after infection. One mouse was not protected and died with high parasitemia by day 6 after infection (Figure 3E), suggesting the lower quality of antibody response.

Conclusions

Immunization of mice with the dead blood stage malarial parasite prepared by treatment with formalin and formulated with highly effective adjuvants like the combination of CpG ODN and Montanide ISA720 can induce protective immune response against challenge infection. The response is consistent with the antibody produced and its elicitation depends on the amount of the parasite and boosters in immunization.

Our future studies will investigate both humoral and cell-mediated immune responses after immunization with four doses of the parasite antigen formulated together with the adjuvants.

Acknowledgements

This research is partially supported by Graduate Studies of Mahidol University Alumni Association, Mahidol University Research Grant, and the Department of Microbiology, Faculty of Public Health, Mahidol University.

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