LOCALIZATION OF *PLASMODIUM FALCIPARUM* ASEXUAL STAGE ANTIGEN BY MOUSE IMMUNE SERA

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ABSTRACT

Malaria is still a major health problem in the world as well as the increased incidence of this disease in the tropical regions. In Indonesia, rapid people movement from/to Java Island is one of reasons in increasing malaria incidence. The use of imprecise dose of antimalarial drugs for medication in community is causing mutation in the parasite genes. This is resulting in parasite resistance to antimalarial drugs. Genetic variation of P. falciparum affects the diversity of clinical symptoms, pathology, transmission characteristic, and human response to antimalarial drugs. An alternative to overcome this problem is using vaccine. However, due to those reasons and also due to the complexity of parasite life cycle the effective and global vaccine is difficult to produce. Through this research we are trying to develop a malaria vaccine for being applied to Indonesia locally. This research is an early step of the main research in malaria vaccine development. This current research is to characterize Plasmodium falciparum asexual stage antigen in order to find out a malaria vaccine candidate for local Indonesia. Previously, 5 BALB/c mice have been immunized with asexual stage antigen of P. falciparum 2300 strain and Freund complete adjuvant. Sera were collected every single week and used to localize the P. falciparum asexual stage antigen by means of indirect immunofluorescent assay (IFA). The results showed that, sera from 3 and 4 weeks post immunization recognized the antigen. Antigens were localized on the surface of late trophozoite, early and late schizont stages, and on the surface of internal and external merozoites. Mouse sera did not recognize ring form and trophozoite stages. It meant that, there was no contamination with ring form and early trophozoite stages during antigen preparation. Hemozoin or malaria pigment was recognized neither by mouse nor by human sera, due to the present of Fe^{++} in hemozoin.

Keywords: Plasmodium falciparum, asexual stage antigen

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INTRODUCTION

Malaria is an infectious disease caused by protozoa of genus *Plasmodium*. Four spesies of *Plasmodium* cause infection in human, *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. Species of *P. falciparum* is the most virulent parasite causing malaria malignant (Faust et al. 1970). In Indonesia *P. falciparum* and *P. vivax* were found more frequently than *P. malariae* and *P. ovale*. These last two species could be found in Irian Jaya and East Nusa Tenggara (Wijayanti et al. 1999). Malaria is still a major health problem in the world as well as the increased incidence of this disease in the tropical regions. Rapid people movement from/to Java Island is one of reasons in increasing malaria incidence in Indonesia Social changes, human migration, natural and man-made changes to the environment contribute to create a complex pattern of malaria transmission. The use of imprecise dose of antimalarial drugs for medication in community is causing mutation in the

parasite genes. This is resulting parasite resistance to antimalarial drugs. Genetic variation of *P. falciparum*, in fact, affects the diversity of clinical symptoms, pathology, transmission characteristic, and human response to antimalarial drugs. Furthermore, mosquito vectors have become increasingly resistant to insecticides. An alternative to overcome this problem is using vaccine (Engers & Godal 1998; Hoffman & Miller 1996; Holder1996; Moorthy et al. 2004). In fact, heterogenicity of individual response to malaria parasite is affected by intensity of parasite exposure, genetic factor of host/parasite, factor ethnic, geographic and parasite strain (Modiano 1996). Due to those reasons and due to the complexity of parasite life cycle causing the effective and global vaccine is difficult to produce. Alternatively, is to produce local malaria vaccine which can be applied to Indonesia locally.

The asexual blood stages of malaria parasite are responsible for the clinical symptoms of malaria.

Therefore, vaccine against these stages is intended to prevent or reduce malaria-related morbidity and mortality by eliminating or reducing the parasite load (Berzins & Perlmann 1996). Merozoite protein is important for erythrocyte invasion and potential for malaria vaccine development. Vaccine against this protein would prevent merozoite invasion of erythrocytes. Therefore, would reduce parasite development and clinical symptoms. (Holder 1996). However, the genetic mutation in parasite strain causing diversity characteristic of each strain (Sherman 1998). Many of the antigens that *P. falciparum* express during their life cycle, particularly the asexual blood-stages, are antigenically diverse. The two major causes of antigenic diversity are allelic polymorphisms and antigenic variation (Anders 1991). Each stage has different antigens that lead to protective immunity and in many cases, these antigens are not expressed at other stages of the life cycle (Hoffman & Miller 1996). Therefore, we need to characterize the antigen herein. The *P. falciparum* asexual stage antigen was isolated from Indonesian isolate of *P. falciparum* 2300 strain, was used to immunize BALB/c mice and mouse sera were used to localize the antigen in the parasite by means of indirect immunofluorescent assay (IFA). This localization of antigen was done to evaluate the antigen preparation. Through this research we are trying to develop a malaria vaccine for being applied to Indonesia locally. This research is an early step of the main research in malaria vaccine development.

MATERIALS AND METHODS

Parasite and in vitro cultivation

Parasite used in this study was *P. falciparum* 2300 strain. Parasites were grown in an in vitro culture system according to the method of Treger and Jensen (1976) with some modifications. Parasites were cultured in a 60 mm Petri dish containing 5% hematocrit of type O human red blood cells (RBCs) in RPMI 1640 medium (Gibco, New York), 0.2% sodium bicarbonate and type O human plasma. The culture was kept in a candle jar and placed in a 37ºC incubator. When the culture reached about 10% parasitemia and majority was ring form stage, culture was then synchronized using 5% sorbitol (Lambros & Vandenberg 1978). Parasites were then cultured for 20 hrs prior to collect the mature schizont-infected red blood cells (Schizont-IRBCs).

P. falciparum asexual stage antigen

Schizont-IRBCs rich culture was harvested, hemolysed with 0.15% saponin to collect merozoites. After washing with phosphate buffer saline (PBS), freed parasites were then sonicated for 5 minutes in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) followed by centrifugation at 5000 rpm and 4°C for 20 minutes. The supernatant was used as antigen. Protein content of this crude antigen was determined according to the Lawry method. This protein was used to immunize BALB/mice.

Immunization

Five BALB/c mice were immunized using 200 μg/ml of *P. falciparum* asexual stage antigen (PfAg) and Complete Freund Adjuvant (CFA), followed by boosting with PfAg and Incomplete Freund Adjuvant (IFA). Immunization was done intraperytonially. Boosting was performed 3 times every single week. Sera were collected from each mouse prior to antibody titration by enzyme-linked immunosorbent assay (ELISA). Another five BALB/c mice were injected with PBS as negative control.

ELISA

Microplate was coated with 200 μg/ml of *P. falciparum* asexual stage antigen in carbonate buffer (0.015 M, Na_2CO_3, 0.034 M $NaHCO_3$, pH 9,6) and incubated overnight at 4°C. Microplate was then washed three times with PBS containing 0.05% Tween 20 (PBST), blocked for 1 hr at room temperature with 200 μl of blocking buffer (10% creamer in H_2O). Mouse sera were diluted at 100X with blocking buffer, and 100 μl of each serum were applied to each well. Incubation was performed at room temperature for 1 hr. After washing 3 times with 0.05% PBST, alkaline phosphatase-conjugated goat anti mouse whole IgG (1:3000), was then applied to each well and incubated as described above. After washing with PBST, 100 μl of Diethanolamine in H_2O as substrate solution were then distributed to each well and incubated in a dark box at room temperature for 30 minutes. The reaction was stopped with 1N H_2SO_4 stop solution. The optical density (OD) values were the read at 490 nm using microplate reader (Bio-Rad Laboratories, CA). The data was analyzed using one way Anova.

Indirect immunofluorescent assay (IFA)

Antigen for IFA was prepared from asynchronous parasite culture (RTS Antigen or RTS Ag) and synchronous parasite culture (MAntigen or MAg). No fixative agents were applied to the antigen. Antigens were dried up at room temperature. Mouse sera were diluted at 10X. *P. falciparum*-infected human sera from several endemic areas of Indonesia (East Nusa Tenggara (NTT), West Nusa Tenggara (NTB) and East Java Provinces (Mlg) diluted at 100X were used as positive

control. Incubation was done at 37°C for 30 minutes, followed by incubation with goat anti mouse FITC-labeled antibody and rabbit anti human FITC-labeled antibody. Fluorescence was observed under UV light microscopy.

RESULTS

Mouse IgG immune response

Immune response in mice is revealed by OD values of antibody titration by ELISA showed that although the OD were relatively low, but the antibody was increase weekly in all mice (Figure 1).
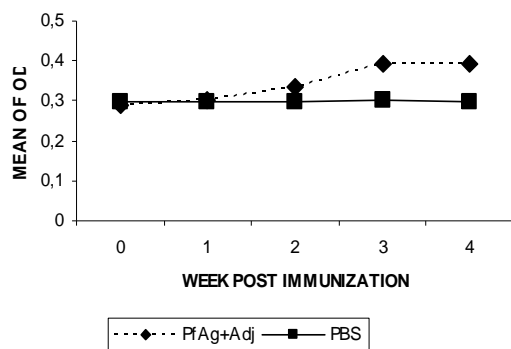

Figure 1. The increase of IgG response in mice immunized with PfAg+Adj compared with negative control (PBS).

Recognition of *P. falciparum* asexual stage antigen

Sera collected at 3 and 4 weeks post immunization were able to recognize *P. falciparum* asexual stage antigen (Table 1). Mouse sera recognized antigen on the surface of late trophozoite, early and late schizont and also internal and external merozoite, but not on the surface of ring form or early trophozoite stages. All samples of human sera as positive control recognized all stages of in vitro intraerythrocytic stages (Table 2).

DISCUSSION

P. falciparum asexual stage antigen was able to induce antibody in mice. Antibody specific to this antigen was produced since first week to fourth week post immunization as shown in Figure 1. Mice immunized with PfAg+Adj showed the response of IgG slightly

higher than that of mice in negative control, and statistically showed a significant difference. In IFA, the relatively low antibody titers of sera collected at 3 and 4 weeks post immunization were able to recognize *P. falciparum* asexual stage antigen. Strong fluorescence were seen on the surface of late trophozoite, early and late schizont, internal and external merozoites stages, but not on the surface of ring form and early trophozoite stages. This result indicated that, the *P. falciparum* asexual stage antigen used to immunize the mice contained only mature stages of intraerythrocytic stages and free of contamination with ring form and early trophozoite stages. This is revealed by retaining positive human serum with ethidium bromide after the process of IFA. The RTS Ag, which was containing ring form, trophozoite and schizont stages, was recognized by all samples of human sera (Figure 3).

Table 1. Recognition of *P. falciparum* asexual stage antigen by mouse sera and human sera from malaria endemic areas of Indonesia

		Fluorescence	
	Sample	MAg	RTS Ag
Mice were immunized with PfAg+Adj	Week 1 post immunization	-	-
	Week 2 post immunization	-	-
	Week 3 post immunization	+	+
	Week 4 post immunization	+	+
Negative control	Week 4 post injection with PBS	-	-
Positive control	LB36 (NTB)	+	+
	P25 (NTB)	+	+
	F46 (NTT)	+	+
	EM1 (East Java)	+	+
	ZM2 (East Java)	+	+

MAg : antigen prepared from synchronous culture
RTS : antigen prepared from asynchronous culture

Table 2: Fluorescence sites of *P. falciparum* reconized by mouse sera and *P. falciparum*-infected human sera

Sample	Fluorescence Sites						
	RF	ET	LT	ES	MS	int M	ext M
Mouse sera	-	-	+	+	+	+	+
Human sera	++	++	++	++	++	+/-	+/-
Negative control	-	-	-	-	-	-	-

RF	:	ring form	Positive control	:	*P. falciparum*-infected human sera from NTT, NTB and East Java Provinces.
ET	:	early trophozoite			
MT	:	mature trophozoite			
ES	:	early schizont			
MS	:	mature schizont			
intM	:	internal merozoite			
extM	:	external merozoite	Negative control	:	PBS-injected mouse sera

The internal and external merozoites some times were found fluorescence sometimes not. Fluorescence on the surface of parasite apparently was cytoplasmic antigens not nuclear antigens, since the antigen was prepared from whole parasites. External merozoites showed an unique typical fluorescence pattern, grape-like fluorescence pattern (Figure 2). This pattern was similar to that of MSP-3 which was recognized by mouse polyclonal antibody against epitope on MSP-3 (Oeuvray et al. 1994). The internal merozoites did not fluoresce strongly as strong as the surface of schizonts. Probably,

due to the dried up antigen (no fixative agent used), therefore the antibody could not penetrate antigen optimally. The *P.falciparum* asexual stages antigen has been recognized by *P. falciparum*-infected human sera from several endemic areas of Indonesia (Arwati & Bumi, 2004; Arwati et al. 2005), so it is potential for local Indonesia malaria vaccine development. The research described here is a very early step in developing malaria vaccine for local Indonesia. It is needed to further characterize the antigen in order to obtain a malaria vaccine candidate for local Indonesia.
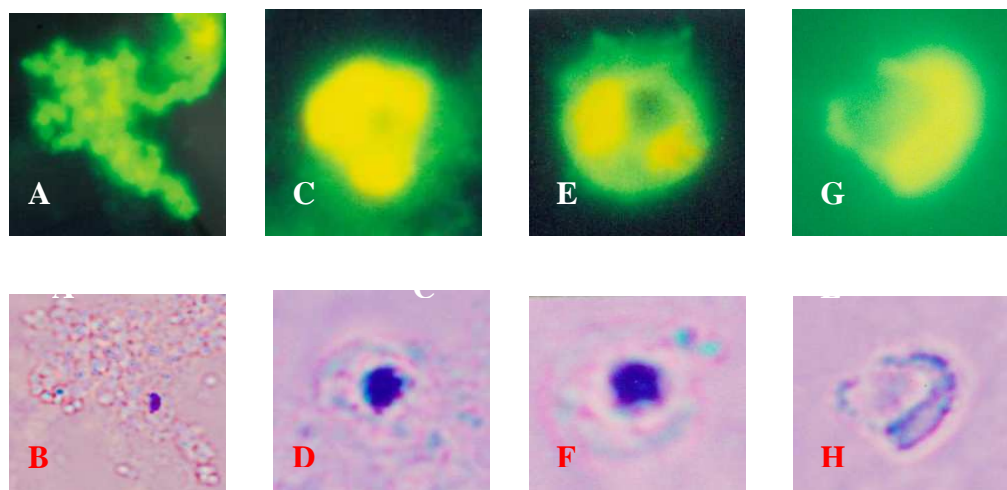

Figure 2. Fluorescence sites of *P. falciparum* recognized by mouse immune sera by indirect IFA. A. Antigen on the surface of external merozoite (*Typical grape-like fluorescence pattern*). B. Phase contrast of Figure A. C. Late schizont D. Phase contrast of Figure C. E. Early schizont F. Phase contrast of Figure E. G. Late trophozoite. H. Phase contrast of Figure G.
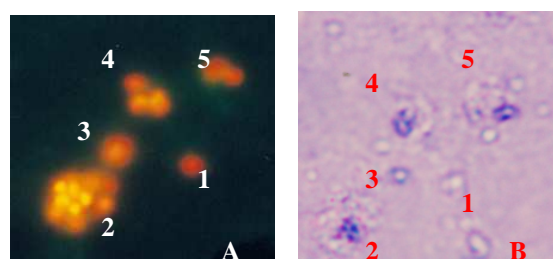

Figure 3. Recognition of RTS Ag by *P. falciparum*-infected human sera from endemic region of Indonesia. A.After IFA process the antigen was restained with ethidium bromide. B. Phase contrast of Figure A. Antigen on the surface of : 1. Ring form, 2. Schizont, 3. Trophozoite, 4. Schizont, and 5. Trophozoite stages.

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