รายงานวิจัยฉบับสมบูรณ์

Naturally acquired antibody profile to *P. falciparum* DBLα in patients with severe and uncomplicated malaria and their relation to rosette formation and binding ability to CD36 of causing isolates.

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สัญญาเลขที่ MRG540382

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บทคัดย่อ

รหัสโครงการ: MRG540382

ชื่อโครงการ: โพรไฟล์ของแอนติบอดีต่อ DBLα ที่เกิดจากการติดเชื้อ *Plasmodium falciparum* ในผู้ป่วยมาลาเรียชนิดรุนแรงและชนิดไม่แทรกซ้อน และความสัมพันธ์ของแอนติบอดีกับการเกิด rosette และความสามารถในการจับกับ CD36 ของเชื้อมาลาเรีย

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บทคัดย่อ : การตอบสนองทางภูมิคุ้มกันแบบจำเพาะต่อการติดเชื้อมาลาเรียที่เกิดจากเชื้อ *P. falciparum* ในคนที่สัมผัสเชื้อเป็นประจำสามารถป้องกันไม่ให้เกิดโรคมาลาเรียที่มีอาการรุนแรงหรือตายได้ แต่กลไกในการป้องกันก็ยังไม่เป็นที่ชัดเจน สำหรับการติดเชื้อในระยะที่อาศัยอยู่ในเม็ดเลือดแดง PfEMP1 เป็นโมเลกุลที่ทำให้เกิด cytoadherence ซึ่งมีความสำคัญในกระบวนการ sequestration ของเชื้อ *P. falciparum* และ Duffy binding alpha (DBLα) เป็นโดเมนที่ทำให้เกิดการ rosetting วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อทดสอบหาโพรไฟล์ของแอนติบอดีต่อ DBLα ในผู้ป่วยมาลาเรียชนิดรุนแรงและชนิดไม่แทรกซ้อน จากการศึกษาพบว่าระดับของ anti-PfDBLα IgG ในพลาสมาของมาลาเรียชนิดรุนแรงสูงกว่ามาลาเรียชนิดไม่แทรกซ้อนอย่างมีนัยสำคัญ (p=0.018) แต่ไม่พบความสัมพันธ์กันระหว่างระดับ anti-PfDBLα IgG กับอัตรา rosetting ทั้งในมาลาเรียชนิดรุนแรงสูงกว่ามาลาเรียชนิดไม่แทรกซ้อน จาก Ig G ทั้ง 4 subclasses ค่ามัธยฐานของระดับ anti-PfDBLα IgG1 ในพลาสมาจากมาลาเรียชนิดไม่แทรกซ้อนมีค่าสูงกว่ามาลาเรียชนิดรุนแรงอย่างมีนัยสำคัญ (p=0.012) ในขณะที่ระดับของ IgG2, IgG3 และ IgG4 ที่จำเพาะต่อ PfDBLα ในพลาสมาจากมาลาเรียชนิดไม่แทรกซ้อนมีค่าค่อนข้างสูงกว่ามาลาเรียชนิดรุนแรง และพบว่า IgG1 มีมากที่สุดทั้งในมาลาเรียชนิดรุนแรงสูงกว่ามาลาเรียชนิดไม่แทรกซ้อน ผลการศึกษานี้ชี้ให้เห็นว่า anti-PfDBLα IgG1 มีผลป้องกันการเกิดมาลาเรียชนิดรุนแรง

คำหลัก : โพรไฟล์ของแอนติบอดี พลาสโมเดียม ฟาลซิพารัม rosetting DBLα

Abstract

Project Code : MRG540382

Project Title : Naturally acquired antibody profile to *P. falciparum* DBL*α* in patients with severe and uncomplicated malaria and their relation to rosette formation and binding ability to CD36 of causing isolates.

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Abstract: Naturally acquired immunity to falciparum malaria protects people routinely exposed to *Plasmodium falciparum* infection from severe disease and death. There is no clear concept about how this protection works. For erythrocytic stages, PfEMP, the cytoadherence molecules of *P. falciparum* plays an important role in sequestration. Duffy binding alpha (DBLα) domain is known to be involve in rosette formation. The objective of this study is to determine the anti-PfDBLα antibody profiles in patients with severe and uncomplicated malaria. As a result, the level of specific anti-PfDBLα IgG in plasma from severe malaria were higher than those from uncomplicated malaria (p=0.018) No correlations of anti-PfDBLα IgG response and rosetting rate in both patient groups were found. Among four subclasses of IgG, the median level of plasma anti-PfDBLα IgG1 from uncomplicated malaria were significantly higher than from severe malaria (p= 0.012). Whereas, the levels of IgG2, IgG3 and IgG4 specific to PfDBLα from uncomplicated malaria were slightly higher than severe malaria. Among the four igG subclasses, the IgG1 against PfDBLα is predominant in both uncomplicated and severe malaria. The findings suggested that anti-PfDBLα IgG1might provide some protection against severe malaria.

Keywords : antibody profile; *P. falciparum* ; rosetting ; DBL*α*

Executive summary

Malaria is one of the most common and important parasitic diseases. Humoral immune responses are believed to be an effective immunity against *P. falciparum*. It has been documented that malaria specific antibodies are associated with protection in both human and animal model (Nielsen *et al*., 2004, Treutiger *et al*., 1992, Moll *et al*., 2007). In endemic area including Africa and Thailand, it has been reported that the predominance of IgG1 and IgG3 individuals to schizont crude extract were associated with protection against *P. falciparum* , while other subclasses and IgM were associated with disease susceptibility (Bouharoun-Tayoun *et al*., 1992). Infection with *P. falciparum* is the most lethal due to the ability to adhere to postcapillary venular endothelium cells (cytoadherence) and the ability to bind to uninfected red blood cells (rosetting). PfEMP1 is a variant surface antigen, strain-specific and highly polymorphic polypeptide of high molecular mass (200 to 350 kDa) which is encoded by a family of *var* genes (Chen *et al*., 2000, Flick and Chen, 2004, Kyes *et al*., 2001). This protein composes of a variable number of adhesive domains of two types, Duffy binding-like (DBL; α, β, γ, δ, ε and X) domains and cysteine-rich interdomain regions (CIDR). The DBLα domain are known as the rosetting domain, while CIDR domain is able to bind with CD36 (Craig and Scherf, 2001). Previously, we have found the distributions of semi-conserved features within the rosetting domain PfEMP1-DBLα and it was close similarity between isolates causing severe and uncomplicated malaria (Horata *et al*., 2009) which were different from those demonstrated in African (Kyriacou *et al*., 2006). The possible explanation is due to different clinical isolates, originated from different geographical areas may mediate different levels of cytoadherences and rosetting. PfEMP1 molecules are potential vaccine candidates that able to generate protective immunity against severe disease. However, no information regarding the natural antibody

response against PfEMP1-DBLα specific domains is available so far in Thai individuals infected with falciparum malaria. The objective of this study is to determine the anti-PfDBLα antibody profiles in frozen plasma from patients with uncomplicated and severe malaria and correlate with their ability to form rosette. The results showed that plasma anti-PfDBLα IgG response in uncomplicated was higher than severe malaria. Of four IgG subclasses, IgG1 against PfDBLα is the predominant subclasses in both uncomplicated and severe malaria, while the similar levels were found in other IgG subclasses. These findings indicated that PfDBLα can induce antibody protection. The understanding on the naturally acquired immune response against DBLα domains of *P. falciparum* might be useful information for malaria vaccine development program.

Objectives

1. To determine whether patients with either uncomplicated or severe falciparum malaria have different specific antibodies response profile against PfDBLα domains.

2. To correlate the anti-PfDBLα antibodies with the ability to form rosette and CD36 binding among isolates causing uncomplicated and severe malaria.

Materials and Methods

1. Study samples

1.1 Stored anonymous plasma obtained from 82 individuals comprising 42 from severe and 37 from uncomplicated malaria and kept at -20oC were studied.

1.2 Twenty stored anonymous plasma from healthy individuals previously collected from Blood Bank. These healthy individuals had no history of malaria exposure.

The study was approved by the ethical committee of Faculty of Tropical Medicine, Mahidol University

2. *P. falciparum* isolates

Fifty two frozen *P. falciparum* isolates including 28 isolates from severe malaria (SM) and 24 from uncomplicated malaria (UCM) obtained from previous study (Horata *et. al*., 2009) and kept in liquid nitrogen were studied. The parasites were thawed immediately and cultivated *in vitro* in malarial culture medium (MCM; RPMI 1640 medium (Gibco Life Technologies, New York, USA) containing HEPES supplemented with 10% human serum from Thai donors, 2mM L-glutamine, 2.5μg/ml gentamicin and 25 mM sodium bicarbonate) in a gas mixture of 5% CO_2, 1% O_2 in N_2 according to previous described (Trager and Jensen, 1976; Horata *et al*., 2009). Briefly, the frozen isolates were removed from liquid nitrogen and quickly thawed at 37°C. Then, the infected red blood cells were transfer to 50-ml centrifuge tubes with a sterile pipette. The volume were measured and 0.1 volume of 12% NaCl were slowly added dropwise and shaking the tube gently. Tube were stand for 5 min. Then, 10 volume of 1.6% NaCl were slowly added, dropwise, swirling the tube. The tubes were centrifuged at 1500 rpm for 5 min. After that, the supernatant was discarded and 10 volume of MCM were added slowly, dropwise, while shaking the tube. Then, tubes were centrifuged at 1,500 rpm for 5 min and the supernatant was discarded. The pelleted infected red blood cells were resuspended in MCM with 15% AB+ serum and transferred to a culture disc and continued culture. Parasite growth was monitored daily by examination of Giemsa-stained thin blood films until the culture reached the pigmented trophozoite stages. At least 1,000 erythrocytes were counted and the parasite stages were recorded and calculated as percent parasitemia. Parasite cytoadherance to CD36 and

rosette formation assay were performed when approximately 70%parasites are trophozoite stages.

3. Cytoadherence to CD36

The static protein-binding assays were determined using purified CD36 (R&D Systems, UK). Briefly, three sets of 10μg/ml CD36 will be spotted onto 35-mm petri-dishes (Nunc, Roskilde, Denmark) and incubated at 37°C for 2 h. in a humidified atmosphere. Dishes were blocked with 1% bovine serum albumin in phosphate buffer saline (PBS) overnight at 4°C, washed with MCM and warmed at 37°C for 30 min. The *P. falciparum* infected RBC were centrifuged and resuspended in MCM, pH 6.8, at 3%parasitemia, 1%hematocrit and 1.25 ml will be added to each dish. Assays were incubated at 37 °C for 60 min with gentle swirling every 15 min. Dishes were gently washed with MCM until no non-adherent RBCs are visible by inverted microscopy. The bound cells were fixed with 2% glutaraldehyde (Sigma, St Louis, MO) in PBS for 20 min and stained with Giemsa (Merck, Poole, UK). The numbers of bound parasitized red blood cell (PRBC) per mm^2 to receptor were counted under light microscope with oil immersion. All assays were done in duplicate. The binding of more than 5 PRBCs/mm^2 were considered significant. The *P. falciparum* laboratory lines, A4 were used as controls.

4. Rosetting assay

Rosetting rate were determined using 100 μl of fresh PRBC suspension mixed with 2 μl of 0.01% acridine orange solution. Ten microliters of the suspension were placed under a 22 X 22 mm cover slip and examined under a fluorescence microscope. Two or three hundred trophozoite stages were counted in duplicate. A rosette was scored if 2 or more uninfected RBC

bound to a single infected erythrocyte. The rosette formation was calculated as the ratio of the numbers of rosette to the total number of parasitized red blood cells.

5. Expression and purification of *P. falciparum* DBLα protein

In brief, the *P. falciparum* were cultured as described above. Total RNA were extracted from malaria parasites when reaching at least 5% parasitaemia as previously described (Kye *et al*., 2000). RNA will be treated with deoxyribonuclease I (DNAse Free, Ambion, UK) to degrade the contaminating genomic DNA. Complementary DNA (cDNA) was synthesized by reverse transcribed RNA with cDNA synthesis kits (Bioline, UK) (RT-PCR) using random hexamers according to the manufacturer. Each cDNA synthesis reaction, a control reaction without reverse transcriptase was done with identical amounts of template. PCR amplification was done according to previously described (Mackintosh *et al*., 2008). The PCR conditions were done using 95^{o}C, 5min and [95^{o}C, 1 min/ 52^{o}C, 1min/ 72^{o}C, 1min/] x30/ 72^{o}C, 5 min. The PCR products were checked by 2% agarose gel. Then the PCR products were ligated into pGEMT easy. Then, the blue white colonies were selected and ligated into pMAL-p5X including the restriction site of EcoRI and Hind III. For protein expression, *E. coli* strain BL21 was used. The transformed cells were grown in Luria-Bertani medium with following supplement; 0.2% glucose, ampicillin (50μg/mL) to maintain the expression plasmid and chloramphenicol (50 μg/mL), to an optical density at 600 nm 0.3 to 0.5 at temperatures 37°C. Each culture was then induced to express the PfDBLα fusion proteins in the presence of isopropyl-β-D-thiogalactoside (IPTG). The samples will be spun and the pellet re-suspended in lysis buffer. The cells were disrupted by sonication. After spinning each fusion protein were purified by chromatography on amylose resin as recommended by the manufacturer (New England Biolabs). After purification,

the protein was stored in the following buffer: 10 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA at -20°C. Protein purity was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis with 10% polyacrylamide and quantity was estimated by a protein assay kit (Bio-Rad, Munich, Germany) as recommended by the manufacturer.

6. Measurement of circulating anti -PfDBL*α* antibody by ELISA

The specific IgG and the subclasses response to PfDBLα domains in plasma were determined by indirect ELISA. The checkerboard titration was done to determine the optimal conditions. All tests were done in duplicate. In brief, wells of microtiter plates (Costar, USA) were coated with DBLα protein suspended in 0.1 M carbonate-bicarbonate buffer pH 9.6. Plates were incubated at 37^{o}C until dry and washed three times with phosphate-buffered saline (PBS) plus 0.05% Tween-20 (PBS-T). The unbound sites were blocked for 2 h at 37^{o}C with blocking buffer containing 2.5% powdered milk in PBS-T. After being washed, plates were added with 50 µl of diluted plasma in 1.25% powdered-milk in PBS-T and incubated for 1 h at 37^{o}C. Plates were washed and incubated with peroxidase-conjugated rabbit anti-human IgG (Dako A/S, Denmark) in 1.25% powdered-milk in PBS-T for 1 h at 37^{o}C for IgG antibody detection. After washing, the color reaction was developed by using *o*-phenylenediamine (OPD) substrate (Dako A/S, Denmark). The optical density (OD) was measured at a wavelength of 492 nm with reference wavelength of 620 nm by an ELISA reader.

The anti-PfDBLα IgG subclasses were determined by ELISA as described above with some modification. After incubation the diluted plasma at 37^{o}C for 1 h, mouse anti-human IgG1, -IgG2 and -IgG3 monoclonal antibodies diluted in 1.25% (wt/vol) powdered-milk in PBS-T were

added. After incubation for 1 h at 37^{o}C, peroxidase conjugate rabbit anti-mouse IgG (Dako A/S, Denmark) diluted in 1.25% powdered-milk in PBS-T will be added and the plate were incubated for 1 h at 37^{o}C. After washing, the color reaction was developed by using OPD substrate (Dako A/S, Denmark) and the OD was measured. Plasma from healthy individuals were used as controls.

7.Data analysis

The plasma antibody response profile to PfDBLα in patients with severe and uncomplicated malaria were compared using Mann-Whitney U test. The correlation of the specific antibody levels in response to DBLα with rosette formation and binding ability to CD36 were tested using Spearman's rank correlation test.

Result

Sample characteristics

The plasma from severe malaria in this study were hyperparasitemia except one with cerebral malaria, and had significant higher parasite count (median = 187,300 parasites/μl) than those with uncomplicated malaria (median=72,590 parasites/μl) (p = 0.000).

Cytoadherance to CD36 and rosetting rate of *P. falciparum* isolates from patients with severe and uncomplicated malaria

The binding abilty to CD36 and rosetting rate were determined in only 52 isolates which could be cultured (28 and 24 isolates from patients with severe (SM) and uncomplicated malaria (UM), respectively). The parasite line; *P. falciparum* clone ItG (originated from

Brazilian IT lineage) was used as a positive control. The numbers of infected erythrocytes (PRBC) of >5 per mm^2 adhered to purified CD36 were considered positive. As the results, 93% and 75% of SM isolates and UCM isolates bound to purified CD36 receptor, respectively. When the numbers of PRBC from SM and UCM isolates adhered to CD36 were compared, no significant differences were found (p = 0.321), however, the mean number of PRBC adhered to CD36 of SM isolates (median = 38.00, IQR= 11.50-142.50) was higher than UCM isolates (median = 33.00, IQR= 1.25-64.25). The correlation between parasite density and ability to adhere to CD36 were fond (p=0.020). For the rosetting rate, 79% of the isolates could form rosettes (23 SM and 19 UCM isolates). The median percent of rosetting rate in SM isolates (median = 9.00, IQR= 3.75-28.50) was lower than UCM isolates (median = 11.00, IQR= 1.25-18.50) with non-significant difference (p=0.512). There is a correlation between parasite density and rosetting rate (p=0.028), but no correlation between the rosetting rate and the binding ability to CD36 was found.

Expression and purification of *P. falciparum* DBLα protein

P. falciparum DBLα protein was successfully expressed in *E coli*. Due to the very low concentration of the cDNA preparation, DBLα was ligated into pGMET easy with the restriction site of EcoRI and HindIII. The target size was 1200 bp. The positive clone was selected and cut with the EcoRI and HindIII. Then, DBLα was confirmed by sequencing. Then the DBLα was ligated into pMAL-p5X and transformed into *E coli*. The positive colonies were selected checked by PCR. The DBLα inserted clones were then cut with EcoRI and HindIII. The colony of clone 22 was pick up and expressed in *E. coli*. The PfDBLα protein was 46.66 KDa. The PfDBLα was purified by amylose column, concentrated and keep at -20^{o}c until use.

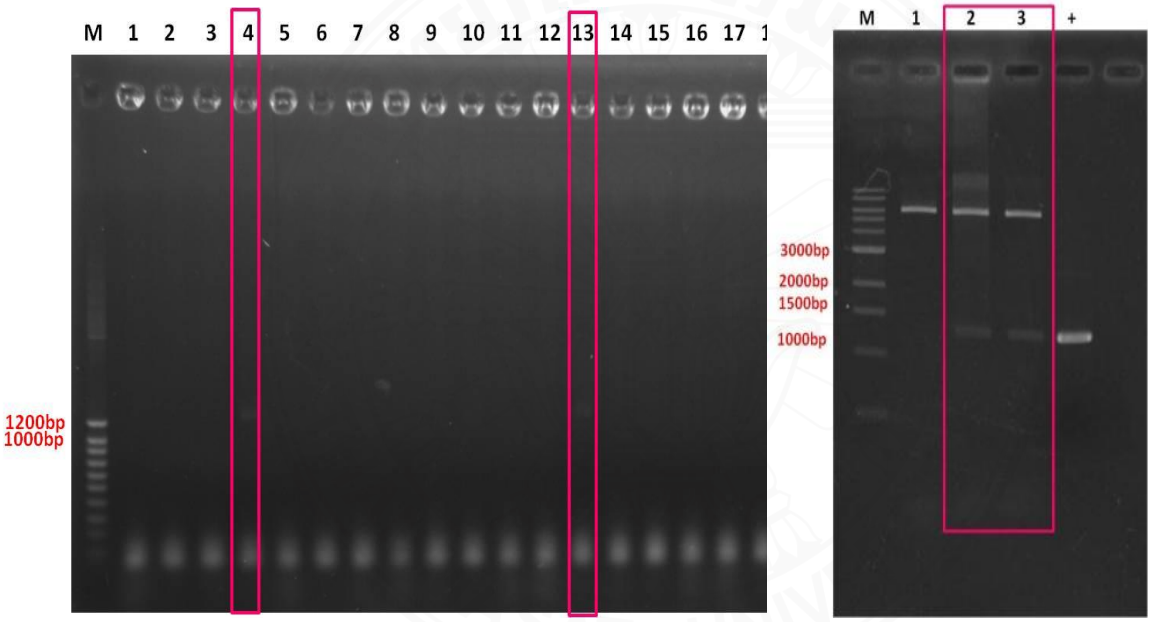

Figure 1 The PCR products of the positive colonies (A). The PCR product cut with EcoRI and HindII (B).

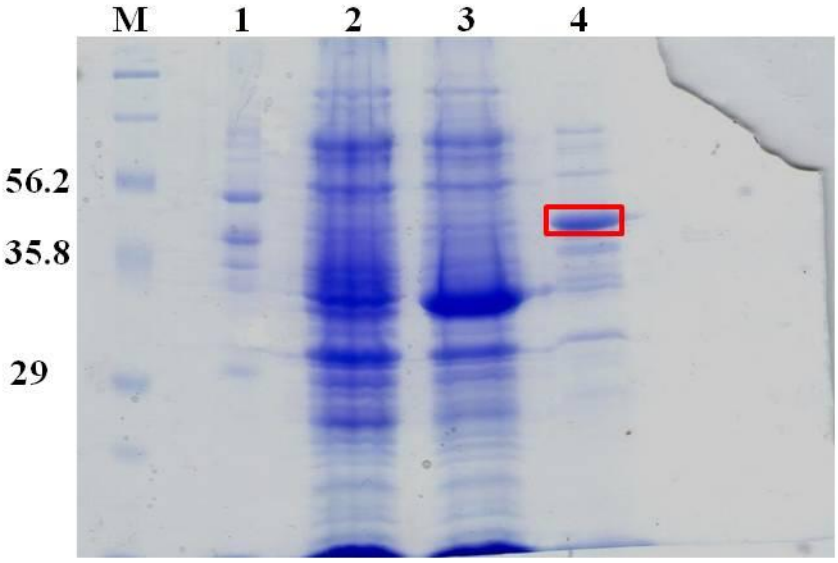

Figure 2 SDS-PAGE of purify *P.falciparum* DBLα; Lane M, molecular weight marker (kDa); Lane 1, RT protein; Lane 2, non-induce pMAL-p5x; Lane 3, induce pMAL-p5x; Lane 4, pMAL-DBLα.

Antibody response to PfDBLα

The optimal conditions of ELISA were studied by checkerboard titration. One hundred microlitter of PfDBLα protein at concentration of 200 ng/ml were used for antigen coating. The optimal dilution of plasma sample was 1:100. The dilution of rabbit anti human IgG conjugated with peroxidase for IgG antibody detection was 1:1000. For subclass detection, mouse anti human IgG1, IgG2, IgG3 and IgG4 were 1:1000, 1:5000, 1:7000 and 1:2000, respectively. The dilution of rabbit anti mouse IgG conjugated with peroxidase was 1:2000.

The antibody responses to PfDBLα were carried out in plasma from 45 severe malaria, 37 uncomplicated malaria and 20 plasma from normal healthy using ELISA. The plasma considered to have the specific IgG anti-DBLα when the optical density (O.D.) values were higher than mean O.D. of normal healthy controls + SD (= 0.315). The specific IgG to PfDBLα in plasma from severe malaria were significantly different from uncomplicated malaria ($p=0.014$). Of these, 40 out of 82 plasma had specific IgG to PfDBLα (18 of 45 and 22 of 37 plasma from severe and uncomplicated malaria, respectively) (Figure 3).

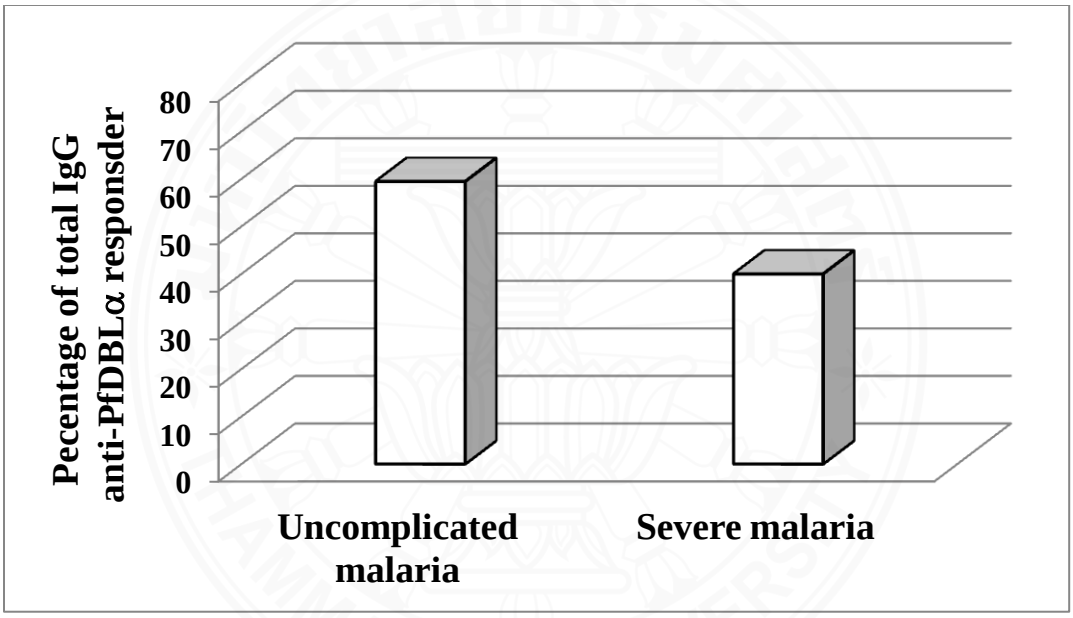

Figure 3 The percentage of plasma anti-PfDBLα IgG responder in from patiwents with severe and uncomplicated malaria.

IgG subclasses including IgG1, IgG2, IgG3 and IgG4 were further investigated in 40 plasma positive for specific IgG to PfDBLα (18 samples from severe and 22 from uncomplicated malaria. The mean level of anti- DBLα IgG subclass levels greater than control plasma plus one standard error (O.D+SE) were considered as positive (0.202 for IgG1; 0.221 for IgG2; 0.205 for IgG3; and 0.174 for IgG4). The percentage of plasma IgG1 in uncomplicated malaria (72.73%) were higher than those in severe malaria (44.44%). While, the percentage of plasma IgG2 were 27.78% and 36.36% in severe and uncomplicated malaria, respectively. The percentage of plasma IgG3 were 38.39% and 27.27% from severe and uncomplicated malaria, respectively. The percentage of plasma IgG4 were 16.67% and 27.27% from severe and uncomplicated malaria, respectively. The median level of plasma anti-PfDBLα for all four subclasses in uncomplicated were higher than those in severe malaria. Among these, only one plasma from

severe malaria had all four subclasses levels. The anti-PfDBLα IgG1 were the predominant subclasses among tested plasma tested, in which 25% of plasma were contained only IgG1 and 77.5% had only IgG1 and IgG3, or coexpressed with other subclasses. Additionally, IgG2 was found to coexpressed with IgG1 and IgG3.

Association between anti- PfDBLα antibody response profiles with cytoadherance to CD36 and rosetting

The plasma anti- PfDBLα antibody response profiles in severe (SM) and uncomplicated malaria (UCM) were compared. The median levels of anti-PfDBLα IgG in UCM plasma was significantly higher than SM plasma (p=0.018). The median levels of anti- PfDBLα IgG1 in UCM plasma was significantly higher than SM plasma (p=0.012). While, no significant differences in the median IgG2, IgG3 and IgG4 levels were found. The significant correlation between anti-PfDBLα IgG and IgG1 levels was found (p=0.008) among SM plasma, While in UCM plasma, a significantly correlation between the levels of anti-PfDBLα –IgG2 and -IgG1 (p=0.006), as well as IgG3 (p=0.044) and IgG4 (p=0.007) was found. The correlation between the ability to bind to CD36 was correlated with the anti-PfDBLα IgG4 levels (p=0.007) in uncomplicated malaria. However, no correlations between PfDBLα specific antibody response and either rosetting rate or the ability to bind to CD36 in both UCM and SM isolates.

Discussion

Naturally acquired immunity targets blood stage *P. faciparum* are important factor of protective immunity. It has been reported that *P.falciparum* specific antibody is short-lived, surviving for only few weeks to months compared to anti-viral antibody but some study show

that *P.falciparum* can also induce and maintain long-lasting parasite specific memory B cell in human (Kaddumukasa *et al*., 2015). The immunoglobulin G (IgG) class has been implicated as important components of such acquired immunity. Antibody specific to PfEMP1 is thought to be an important key of the protective immunity for the patient infected with *P. falciparum* that takes years to develop the antibody due to its polymorphic and it can switch expression among different variants. PfDBLα is one of antibody target. Our study show that only 48.78% of infected with *P. falciparum* plasma contained anti- PfDBLα IgG, which might be possible that, based on large repertoire PfEMP1-DBLα variants, each infection is accompanied by different PfEMP1-DBLα (Oguariri *et. al*., 2001). This observation is in accordance to the previous report in Thailand that 44.4% of sera from patients infected with *P. falaciparum* had anti-*P. falciparum* IgG (Tangteerawatana *et.al*., 2001). A significant difference in anti-PfDBLα IgG between severe and uncomplicated malaria were observed, with higher in uncomplicated malaria. The finding indicated that PfDBLα could induce protective antibody response . The anti-PfDBLα IgG1 was the predominant subclasses. IgG2 was found to coexpressed with IgG1 and IgG3. This is in line with the report in Africa that , IgG1 and IgG3 antibodies were the predominant subclasses which protected *P. falciparum* infected African adults (Bouharoun-Tayoun and Druilhe, 1992). In additional, IgG specific to PfEMP1 are believed to contribute to the regulation of parasite densities (Giha et al., 2000). The balance of antibody subclasses and the level of antibodies are importance in antibody-mediated protection against malaria. The antibody protection success, if there are sufficient levels of antibodies of the correct specificity and appropriate subclass. The possible mechanism of protection is that the potential antibody could induce phagocytic clearance, reduce cytoadherence, inflammation and parasite burden (Bouharoun-Tayoun and Druilhe, 1992, Dodoo D *et al*. 2001). But, no significant correlation between the specific anti-

PfDBLα antibody and the rosetting rate was found in this study. Additionally, the median number of PRBC adhered to CD36 in severe malaria isolates was higher than uncomplicated malaria isolates as shown in the previous report (Chaiyaroj et al., 1996), indicating that the due to the variation in PfDBLα domain, this is the evasion strategy of *P. falciparum* to avoid from the host immune system by hiding from phagocytosis, enhancing parasite growth, survival leading to high parasitaemia *in vivo* and increase microvascular obstruction of the blood flow, mediating acute inflammation (Kaul *et al*., 1991, Miller *et al*., 2000, Rowe *et al*., 2000).

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