การค้นหาโปรตีนแกมมีโตไซท์ของพลาสโมเดียม ฟัลซิพารัม ซึ่งกระตุ้นการสร้างแอนติบอดี

CHARACTERIZATION OF GAMETOCYTE PROTEINS INDUCING NATURAL ANTIBODIES IN *PLASMODIUM FALCIPARUM* INFECTION

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บทคัดย่อ

มาลาเรียมีวงจรชีวิตที่ซับซ้อนโดยเฉพาะระยะแกมมีโตไซต์ ซึ่งเป็นระยะที่ติดต่อสู่ผู้อื่นได้โดยพาหะยุงก้นปล่อง การเพิ่มพูนความรู้ด้านโปรตีนของระยะนี้ที่มีบทบาทต่อการตอบสนองภูมิคุ้มกัน จะนำไปสู่การพัฒนาวัคซีนป้องกันการติดต่อ ซึ่งสามารถลดอัตราการติดเชื้อนี้ได้ งานวิจัยนี้เน้นศึกษาการติดเชื้อของพลาสโมเดียมฟัลซิพารัม เนื่องจากพบได้มากที่สุดและมีอันตรายถึงขั้นชีวิต น้ำเหลืองของผู้ป่วยที่ติดเชื้อมาลาเรียนี้ 97 ตัวอย่างถูกนำมาประเมินแอนติบอดีต่อโปรตีนของไซซอนต์และแกมมีโตไซต์ ด้วยวิธี IFA และ ELISA พบว่ามากกว่า 66% ของตัวอย่างมีแอนติบอดี ด้วยความก้าวหน้าของเทคโนโลยีทั้งด้านจีนโนมิคและโปรติโอมิค จึงได้ทำการสังเคราะห์โปรตีนที่จำเพาะขึ้น โปรตีนสมมุติฐานของแกมมีโตไซต์ที่ถูกสังเคราะห์โดยวิธีปราศจากเซลล์นั้น ถูกนำมาทดสอบปฏิกิริยากับน้ำเหลืองตัวอย่าง ซึ่งมีค่าไตเตอร์สูงและมีผล IFA ที่แตกต่างกัน พบว่าทำปฏิกิริยาอ่อนๆ กับตัวอย่างที่มีผลลบต่อแอนติบอดีของไซซอนต์ โดยมีค่าไม่เกินสองเท่าของตัวอย่างที่ไม่ติดเชื้อ อย่างไรก็ตามผลของปฏิกิริยากับโปรตีนแกมมีโตไซต์ที่ถูกย่อยเชิงกล (sonication) มีค่าสูงมากถึง 4 เท่าของตัวอย่างไม่ติดเชื้อ จึงสรุปได้ว่าโปรตีนที่สังเคราะห์ด้วยวิธีปราศจากเซลล์ไม่น่าจะมีคุณสมบัติในการกระตุ้นการผลิตแอนติบอดีตามธรรมชาติได้ดี แต่โปรตีนที่กระตุ้นการผลิตแอนติบอดีนี้ยังคงมีอยู่ในโปรตีนที่ทำปฏิกิริยาภายในโปรตีนที่ถูกย่อยเชิงกล การศึกษาอย่างต่อเนื่องเกี่ยวกับโปรตีนที่ทำปฏิกิริยาภายในโปรตีนที่ถูกย่อยเชิงกลอาจนำมาซึ่งการพบตัวแทนวัคซีน

คำสำคัญ: มาลาเรีย พลาสโมเดียม ฟัลซิพารัม แกมมีโตไซต์ แอนติบอดี

ABSTRACT

Within the complex malaria cycle, gametocytes are responsible for transmission via the *Anopheles* mosquito. Increase knowledge of gametocyte proteins towards immune response will be used for the development of transmission blocking vaccine thus decreasing the rate of malaria infection. Focusing on *Plasmodium falciparum* infection, the most prevalent and fatal malaria species, 97 infected anti-malaria sera were evaluated for antibodies against schizont and gametocyte antigen by immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA). More than 66% were found positive by both assays. With the progress of both genomic and proteomic technologies, the expression or synthesis of specific proteins is possible. Cell-free expressed theoretical gametocyte proteins further reacted with high titer anti-malaria sera of either positive or negative IFA results indicated weak reactivity with anti-malaria sera negative for schizont antibody, no more than 2 times the optical density of uninfected plasma. On the other hand, that of crude gametocyte protein sonication produced a remarkably strong reaction, as high as 4 times of that of uninfected. These tested cell-free expressed proteins does not possess good characteristics for inducing natural antibody production but possible antigens within the sonicated crude gametocyte proteins are present as seen in their reaction. Continual investigation into reactive proteins in crude sonication may lead to a possible vaccine candidate.

KEYWORDS : Malaria; *Plasmodium falciparum*; Gametocyte; Antibody

1. Introduction

Malaria is the world's most important tropical disease. It is the leading cause of deaths and diseases worldwide. According to the World Health Organization, 47% of the world's population, a total of 2,400 million people, lives in malaria transmitted areas consisting of about 101 countries. As many as 300 to 500 million people are infected with malaria annually (World Health Organization, 2006) of whom 700 to 2.7 million die. With the increasing rate of morbidity and mortality, malaria has caused an enormous impact on both the economic status and well being of the subtropical and tropical areas (World Health Organization, 2006).

This endemic disease is caused by protozoa from the genus *Plasmodium*. *P. vivax*, *P. falciparum*, *P. ovale* and *P. malariae* are the only four known species which cause human malaria (National Center for Infectious Disease, 2004). Of these four, *P. vivax* and *P. falciparum* are the most prevalent in the world. The latter causes cerebral malaria, a more severe and often fatal (Day, 1999).

The *Plasmodium* parasite's life cycle is complex, having both asexual and sexual stages, alternating between the vertebrate host, human, and the invertebrate host, mosquito of the genus *Anopheles* (Carter and Ranford-Cartwright, 1998). The sporozoites from the mosquito vector are transmitted to humans by a single bite. This initiates the beginning of the exoerythrocytic stage also known as the incubation period (National

Center for Infectious Disease, 2004). When the exoerythrocytic schizont is fully developed, the liver cell bursts, releasing merozoites into the host's blood stream. This then starts the human blood stage or erythrocytic stage (Fujioka and Aikawa, 1999), the easiest detectable stage. Some patients have only asexual stages consisting of rings, trophozoites, and shizonts; whereas a smaller group has sexual stages, gametocytes, present in their erythrocyte. The latter stage plays a key role in the transmission of malaria. Recent research has found that specific antibodies towards gametocyte lessen the development of oocyst within the mosquito thus causing a reduction in transmission (Tsuboi and others, 2003). This immunity is called transmission blocking immunity and primarily consists of antibodies which are specific to antigens involving the fertilization of the parasites (Drakeley and others, 2006). Currently, there is no malaria vaccine approved for human use (National Center for Infectious Disease, 2004) or a definite method of preventing this disease from spreading. So far, there is not enough information on which proteins or antigens are specific antigen to these antibodies(Sutherland, 2009); therefore, this study intends investigate and characterize selected gametocyte proteins synthesized by wheat germ cell-free protein production system as antigens inducing natural antibodies prevalent in *P. falciparum* infected patients in Thailand.

2. Methodology

1. Plasma collection

1.1 Normal plasma collection: Blood were obtained from non-malaria infected donors of random blood groups during the year 2007. Plasma were separated from their erythrocytes by centrifugation and inactivated before conducting any further experiments. A total of 15 normal plasma have been obtained. Plasma were stored at -20 ^{o}C.

1.2 Anti-malaria plasma collection: Blood from *Plasmodium falciparum* infected donors, admitted to the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University in the year 2005, were used. The blood sample collected had been heparinized. Anti-malaria plasma were collected by centrifugation and further inactivated before conducting any further experiments. Anti-malaria plasma were stored at -20 ^{o}C.

2. Schizont and gametocyte enrichment

The cultured schizonts or gametocytes with high parasitemia were adjusted to 20% hematocrit and over laid on top of freshly prepared 5 ml Percoll® [9 (Percoll® and 10X RPMI) : 1 1X RPMI], 60% Percoll® for schizont separation and 47% Percoll® for gametocyte separation. Then tubes were centrifuged at 2,800 rpm for 25 min at 10 ^{o}C for schizont and 27 ^{o}C for gametocyte separation. The thin interface was carefully collected and washed thrice with 1X RPMI 1640 (Gibco™). The pellets were further processed for IFA and ELISA.

3. Wheat germ cell-free protein production

Gametocyte genes were selected from Plasmodb.org which had high expression during gametocyte stage. Most of them translate to hypothetical proteins. Plasmids containing selected gametocyte genes were processed by PCR (94 ^{o}C X 2 min, 98 ^{o}C X 10 sec, 55 ^{o}C X 30 sec (35 cycles), 72 ^{o}C X 3 min (2 kb/min), 72 ^{o}C X 7 min, and 20 ^{o}C hold) by Taq polymerase. PCR product was checked by 1.0% Agarose gel electrophoresis. Transcription of genes for mRNA was done and precipitated for further synthesis. Wheat germ cell-free protein was synthesized using bilayer method (Sawaski and others, 2002). Upper buffer was added into each well of round bottom 96 well plates. The mRNAs of gametocyte proteins were resuspended in lower buffer avoiding bubbles. This solution is then loaded to the bottom of each well by not disturbing the interphase of the 2 buffers. The plates were sealed and incubated at 17 ^{o}C for 16 hr or overnight. The plates were later kept at – 80 ^{o}C.

4. Immunofluorescence assay

The schizont and gametocyte enriched pellet was diluted with phosphate saline buffer (PBS) [1:50] and spotted on 24 multiwell slides. Once the slides were dry, they were fixed with cold acetone and blocked with 5% Fetal Calf Serum (FCS) in PBS, 1 hr in the dark at 37 ^{o}C. Anti-malaria and normal plasma with 1:25 dilution were added. After 30 min similar to the previous condition, the slides were washed thrice before Rabbit Anti-Human IgG FITC with 1:50 dilution were added. This was also placed in the dark at 37 ^{o}C for 30 min. Before 50 µl of 80% Glycerol in PBS was placed on the slide for the coverslip to adhere, the slides were washed thrice with cold PBS. Once the coverslip was placed on, the slides were stored in moist chamber at 4 ^{o}C for no more than a week for further evaluation under fluorescent microscope.

5. Enzyme-linked immunosorbent assay

10 µg/ml of non-malaria infected erythrocyte protein, gametocyte proteins, schizont proteins in coating buffer, and cell-free expressed proteins were diluted [1:50] were used as antigen to coat each well of 96 well Nunc-immuno ™ plates overnight at 4 ^{o}C. Excess antigen buffer were removed and blocked with blocking buffer for 2 hr at room temperature. Then the wells were washed with TBS-T (0.05% Tween-20 in PBS) thrice. The anti-malaria and normal plasma were diluted to 1:100 in PBS and added to each well. Plates were incubated overnight at 4 ^{o}C. After washing thrice, Horseradish Peroxidase-conjugated Rabbit anti-Human IgG [1:2000] was added and incubated for 1 hr at room temperature. After washing with TBS-T thrice, 2,2'azino-di-(3-ethylbenzthiaolin sulfonic acid) (ABTS) peroxidase substrate solution was added and incubated for another 20 min in the dark at room temperature. Once the time period was reached, the enzyme reaction was measured by automated microplate reader at 405 nm.

3. Results

Microscopic examination of malaria infection was done by medical technologist at the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University. Schizont parasitemia were observed in thin blood sample films, whereas the presences of gametocytemia were evaluated by observing the stained thick

blood sample films under light microscope. All blood samples (100%) had *Plasmodium falciparum* schizont stage infected erythrocytes, but gametocyte infected erythrocytes were presented in only 50% of the samples.

Anti-malaria antibodies, naturally induced by malaria infection, were evaluated for the presence of antibodies by two assays immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA).

Table 1. Percentage of plasma positive for *Plasmodium falciparum* schizont and gametocyte antibodies by IFA and ELISA.

Assay	Schizont Antibodies	Gametocyte Antibodies
IFA	66	67
ELISA	79	84

Although all blood samples had schizont infected erythrocyte, but evaluation of naturally induced anti-malaria schizont antibodies from 97 *Plasmodium falciparum* infected plasma by IFA showed that only 66% of the plasma were positive for anti-schizont antibodies, Table 1. ELISA confirmed the presence of such antibodies to 79% by using sonicated schizont antigens. Determination of ELISA cutoff point, 0.4, was based on the mean optical densities (OD) of 20 normal plasma, $\overline{X}$ = 0.02, with the addition of 1 standard deviation (SD), 0.02, at 68% confidence.

Anti-gametocyte antibody positive samples evaluated by IFA showed bright green fluorescence on the gametocyte infected erythrocytes. Anti-gametocyte antibody by IFA showed 67% of the samples, Table 1, whereas gametocytemia had only 50%. The presence of anti-gametocyte antibodies in the plasma showed 84% positive by ELISA. Any sample with OD higher than 68% confidence value, $\overline{X}$ + 1SD, of the infected sera was considered positive.

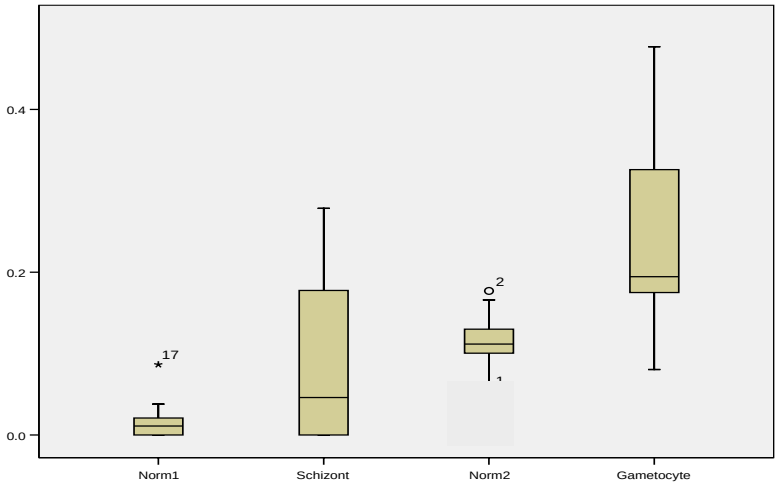

Figure 1. Comparison of antibody to malaria antigens in normal plasma and malaria infected plasma shown by ELISA. Antigens were prepared from schizont and gametocyte. Norm 1 and Norm 2 were reacted with schizont or gametocyte antigens respectively. Data are shown in median (horizontal bars), interquartile ranges (box plots), maximum and minimum (upper-lower lines).

Antibodies against schizont antigens analyzed by ELISA, shown in Figure 1, indicated that the range of anti-schizont antibody level was wide, and some were similar to those of normal plasma. The antibody level range in normal plasma was narrow and the median is centered. Almost all plasma had high anti-gametocyte antibodies than that of the normal plasma, Norm 2. Only a few plasma samples had a similar OD level to that of the normal plasma. The range of anti-gametocyte antibodies was broad.

All 157 cell-free expressed gametocyte proteins were reacted with 5 pooled or combined anti-gametocyte antibody positive plasma, having high OD levels. 13 gametocyte proteins with the highest OD values, protein number 83, 90.2, 96, 128, 150.2, 154, 160, 162, 165, 167, 178, 184, and 185 which were translated from gene codes PF08_0077, PF13_0220, PF10_0365, PFL 1265c, PFI 1345c, PF14_0672, PF10_0365, PF10_0190, MAL7P1.35, PF08_0017, PFI 1315c, PF13_0298, and PF11_0456, respectively, were selected to react with the selected 6 anti-malaria plasma. The SD of normal plasma OD against each cell-free expressed protein was nearly half the OD of the mean normal plasma. These positive reactions were very weak and not convincingly positive. Plasma numbers 74 and 35 had reaction with all the selected gametocyte expressed protein. These two infected plasma were negative for anti-schizont antibodies by IFA. Protein 162, 167, 178, 184, and 185 also had weak reactivity with plasma number 77 and 94. Only 4 proteins, 128, 160, 184, and 185, had high reactivity of OD higher than mean of normal plasma with the addition of 2 S.D.

All four infected plasma were positive for anti-schizont and anti-gametocyte antibodies by ELISA, but anti-malaria serum number 74, 35, and 94 were negative for anti-schizont antibodies by IFA. Plasma number 91 is also negative for anti-schizont antibodies by IFA but produced no reaction. Anti-gametocyte antibody present in the plasma does not always react with the gametocyte proteins.

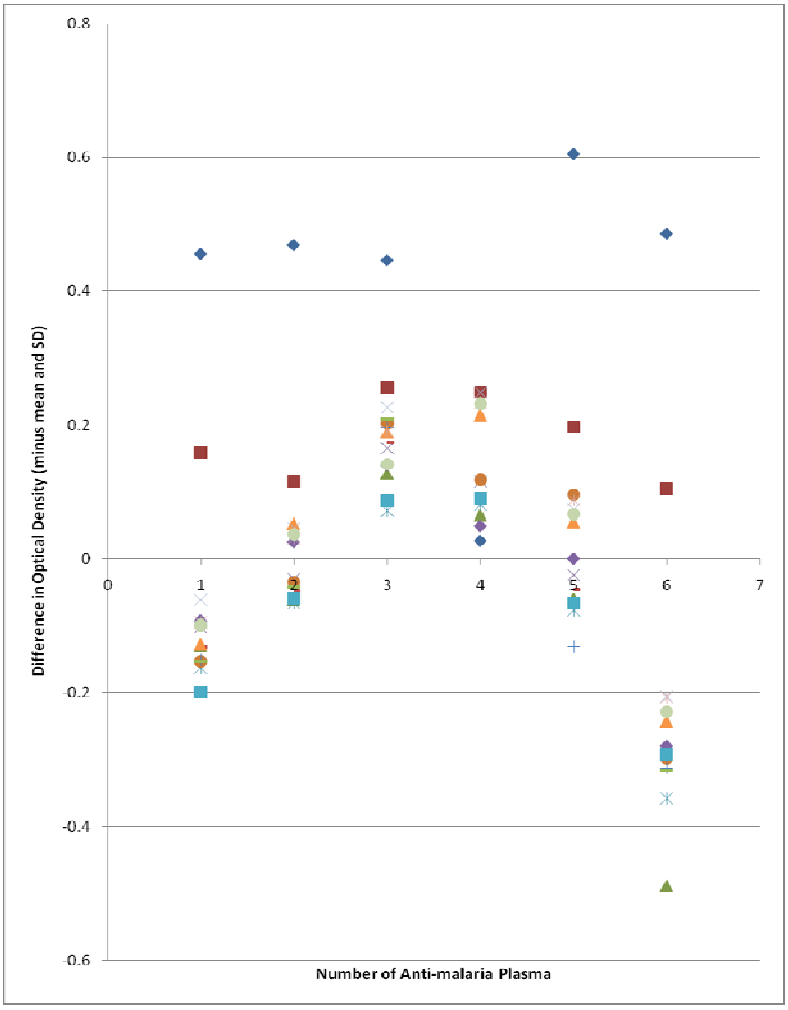

Figure 2. Difference in optical densities of anti-malaria antibodies of the 6 plasma (1 = no. 64, 2 = no. 77, 3 = no. 74, 4 = no. 35, 5 = no. 94, 6 = no. 91) with sonicated gametocyte proteins (◆), sonicated schizont proteins (■), protein 83 (▲), protein 90.2 (×), protein 96 (✳), protein 128 (●), protein150.2 (+), protein 154 (▪), protein 160 (▪), protein 162 (◆), protein 165 (■), protein 167 (▲), protein178 (×), protein 184 (✳), and protein 185(●). All OD values were subtracted by mean OD of normal plasma reacting with the corresponding protein tested and 1 SD.

Difference in OD of the reaction of anti-malaria antibodies with sonicated schizont proteins, sonicated gametocyte protein, and expressed gametocytes, Figure 2, showed that the OD of the reaction with gametocyte sonicated protein is higher than the OD reaction with selected expressed gametocyte proteins by 4 folds. Plasma number 4 was the only plasma having OD reaction to expressed gametocyte proteins higher than the OD reaction to expressed gametocyte proteins.

4. Conclusion

This study focuses on naturally induced antibodies during *Plasmodium falciparum* infection. Examination of anti-malaria antibodies to schizont antigens and gametocyte antigens was done by two assays, IFA and ELISA. Percentages positive for anti-schizont antibodies were slightly lower than parasitemia but that of anti-gametocytemia were higher. This suggests that gametocyte detection by light microscopy, the gold standard should not be used for gametocytemia. Some other molecular means of detection may be the solution.

Comparison of anti-schizont and anti-gametocyte antibody presence showed that *P. falciparum* infection produced higher levels of anti-gametocyte antibodies even though the gametocyte density is greatly lower than schizont density. The production of antibodies does not depend on the density of the parasite. Classification of the anti-malaria plasma revealed that most of the plasma were positive for both anti-schizont and anti-gametocyte. Cell-free expressed gametocyte proteins showed little reaction with the selected anti-malaria plasma, thus indicating that these proteins possess little characteristics as a prominent antigen. Two plasma had weak reactions with all the selected proteins. Some proteins reacted with 4 plasma that were of different classification.

The selected proteins slightly induced antibody , however, the antigen producing high reaction to with the gametocyte antigen is still present and remains anonymous. The OD of the infected plasma to sonicated gametocyte protein is 4 times higher than that to the gametocyte proteins. Certain gametocyte surface antigens such as Pfs230 and Pfs48/45 have been found to induce antibody production (Sutherland, 2009); however, further research to pin point the protein with natural antibody production properties should be carried out. The success of this find will contribute to the development of new and possible transmission blocking vaccine.

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