Monoclonal Antibodies Against Malarial Antigens Using Magnetic-Enriched Hemozoin from *Plasmodium falciparum*-infected Erythrocytes as an Immunizing Agent
โมโนโคลนัลแอนติบอดีต่อแอนติเจนของเชื้อมาลาเรียโดยใช้ฮีโมโซอินเป็นสารกระตุ้นภูมิคุ้มกัน

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ABSTRACT

The purpose of this study was to leverage the unique paramagnetic properties of hemozoin to obtain hemozoin-associated proteins from parasite-infected erythrocytes for monoclonal antibody production. The monoclonal antibodies (MAbs) against hemozoin-associated proteins were tested for specificity and reactivity by western blotting and immunofluorescence assay. Two MAbs are the highlight, 13C7 and 10A2 recognized a 40 kDa *Plasmodium falciparum* protein and 55 kDa histidine-containing protein, respectively. The reactivity of 13C7 was observed on the margin surrounding the parasite, whereas 10A2 was diffuse over the entire infected erythrocyte during the ring, trophozoite, and schizont stages.

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อใช้ประโยชน์จากสมบัติพิเศษพาราแมกเนติกของฮีโมโซอินมาเตรียมโปรตีนที่เกี่ยวข้องกับฮีโมโซอินจากเม็ดเลือดแดงที่ติดเชื้อมาลาเรียสำหรับการผลิตโมโนโคลนัลแอนติบอดี เมื่อนำโมโนโคลนัลแอนติบอดีที่ผลิตได้มาทดสอบความจำเพาะและปฏิกิริยากับโปรตีนที่เกี่ยวข้องกับฮีโมโซอินด้วยวิธี western blot และ immunofluorescence พบว่า โมโนโคลนัลแอนติบอดีจำนวน 2 โคลน มีความจำเพาะต่อโปรตีนที่เกี่ยวข้องกับฮีโมโซอิน โดยโมโนโคลนัลแอนติบอดี 13C7 และ10A2 มีความจำเพาะต่อโปรตีนของเชื้อ *Plasmodium falciparum* ขนาด 40 กิโลดาลตัน และ 55 กิโลดาลตันที่มีกรดอะมิโนฮิสทิดีนเป็นองค์ประกอบ ตามลำดับ เมื่อทดสอบการเกิดปฏิกิริยาของ 13C7 และ 10A2 กับเม็ดเลือดแดงที่ติดเชื้อมาลาเรีย พบว่าเม็ดเลือดแดงซึ่งย้อมด้วย 13C7 ติดสีเรืองแสงในส่วนขอบล้อมรอบตัวเชื้อ ในขณะที่ย้อมด้วย 10A2 ติดสีเรืองแสงแพร่กระจายไปทั่วเม็ดเลือดแดงที่ติดเชื้อ ทั้งในระยะ ring, trophozoite และ schizont

Keywords: Malaria, Hemozoin, Monoclonal antibody

คำสำคัญ: มาลาเรีย ฮีโมโซอิน โมโนโคลนัลแอนติบอดี

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Introduction

All *Plasmodium* parasites produce a 'malarial pigment' that is known as hemozoin (Egan, 2008). Hemozoin is the end product of hemoglobin degradation within the food vacuole of a malarial parasite. Hemozoin is produced throughout the intraerythrocytic development of the parasite, and then released together with the merozoites after the rupture of the infected erythrocyte (Noland et al., 2003; Wood, Eaton, 1993). Highly toxic free heme is released as a byproduct of hemoglobin degradation in the acidic food vacuole. Hemozoin crystals are quickly formed as a result of heme detoxification in a process known as biomineralization or biocrystallization (Egan et al., 2001).

Hemozoin is important for malaria detection, because it can be used as a biomarker of all blood-stage malaria parasites, including the ring stage. Studies in the use of hemozoin to detect parasitized erythrocytes have primarily focused on the unique optical properties of hemozoin. Intracellular hemozoin has been detected in parasitized erythrocytes using polarized light microscopy even when the level of parasitemia is very low (Lawrence, Olson, 1986). *P. falciparum* hemozoin pigments were successfully detected in the blood of asymptomatic pregnant women by laser desorption mass spectrometry (LDMS) (Nyunt et al., 2005). Moreover, hemozoin crystals in malaria-infected patient blood were detected by flow cytometry (Grobusch et al., 2003; Rebelo et al., 2013).

Hemozoin crystals inherently exhibit paramagnetic properties. A magneto optical test (MOT) instrument was evaluated for its ability to detect *P. falciparum* in infected erythrocytes using *in vitro* parasite cultures. That test could detect malarial parasite populations as small as 40 parasites per µl of blood (0.0008% parasitemia) during the ring stage, and less than 10 parasites per 1 µl of blood (0.0002% parasitemia) during the schizont stages (Orban et al., 2014). In another study, the hemozoin pigment in human blood with 0.2% parasitemia could be detected by a nuclear magnetic resonance (NMR) device (Karl et al., 2011). Hemozoin has not been well studied relative to the applicability of its paramagnetic properties in serology.

Objective of the study

The aim of this study was to employ the paramagnetic properties of hemozoin to obtain hemozoin-associated proteins for monoclonal antibody production. Hemozoin was enriched from malaria-infected erythrocytes using a simple magnetic separator, after which the obtained hemozoin-associated proteins were used as the antigens for the immunization of mice. Our results revealed two monoclonal antibodies (MAbs) that recognized hemozoin-associated proteins.

Materials and methods

Hemozoin preparation

Hemozoin was prepared by a magnetic separator as previously described (Kim et al., 2010; Ribaut et al., 2008) with some modifications. Briefly, *P. falciparum* clone 3D7-infected human erythrocytes containing mixed-stage parasites were kindly supplied by the late Dr. Sastra Chaotheing, Protein-Ligand Engineering and Molecular Biology Laboratory, National Center for Genetic Engineering and Biotechnology, Pathum Thani, Thailand. The infected erythrocytes were lysed with 1% saponin. Following three washes with PBS, the cells were resuspended in phosphate-buffered saline (PBS) and subjected to three rounds of freeze-thaw lysis with liquid nitrogen. Prior to enrichment, an LS column placed in the magnetic field of a VarioMACSTM Separation Unit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was equilibrated with PBS. Fifteen ml of crude lysates was then loaded on the top of the LS column. The unwanted particles flowed through, leaving the enriched hemozoin on the top of the LS column. Following three washes in PBS containing 0.1% (v/v) Tween-20 (PBST), the magnetic separator was removed and the enriched hemozoin was eluted with 5 ml PBS. The enriched hemozoin was recovered by centrifugation at 12,500 g for 10 min and then resuspended in PBS. The heme content was measured by the dissolution of hemozoin into monomeric heme in a reaction solution of 20 mM NaOH/2% SDS with incubation for 1 h at room temperature (RT). Monomeric heme, which has a molar extinction coefficient of 1×10^5 at 400 nm, was determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The heme content measured at 400 nm was calculated using the Beer-Lambert equation, as follows: $c = A/(E*b)$, assuming c to be the concentration in moles/liter or molarity (M), A to be the absorbance in absorbance units (AU), E to be the molar extinction coefficient, and b to be the path length in cm. The 26-29 nmol of heme can be recovered from 25 μg of a hemozoin preparation (Fitch, Kanjananggulpan, 1987; Sullivan, et al., 1996).

Morphologic study of *P. falciparum* hemozoin by scanning electron microscopy (SEM)

The enriched *P. falciparum* hemozoin was suspended in distilled water, and the aggregates of enriched hemozoin were dispersed using an ultrasonic bath. The dispersed suspension was subsequently deposited with a dropper onto 5 mm glass slides. After drying the sample, the glass slide was coated with gold (JFC-1200 Fine Coater; JEOL Ltd., Tokyo, Japan). The loaded glass slide was then characterized by scanning electron microscopy (JSM-6301F; JEOL, Ltd.).

Immunization and monoclonal antibody production

Prior to immunization, the enriched *P. falciparum* hemozoin preparation was sonicated three times for 10 s each using a probe sonicator (Branson Digital Sonifier 450; Branson Ultrasonics, Danbury, CT, USA). Three female BALB/c mice (National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand) were immunized intraperitoneally with 50 μg of enriched hemozoin emulsified in Complete Freund's Adjuvant (Sigma-Aldrich Corporation, St. Louis, MO, USA), followed by boosts with 20 μg of the same antigen emulsified in Incomplete Freund's Adjuvant (Sigma-Aldrich) at 3-week

intervals. After the fifth immunization, the antibody titer was assessed by slot immunoblot assay. The mouse that exhibited the highest antibody titer was further boosted with an intravenous tail vein injection of 50 µg of the same antigen without adjuvant. Three days later, the mouse was sacrificed and spleen cells were used for fusion with mouse myeloma cells.

MAbs were prepared by the fusion of spleen cells from the immunized mouse with X63-Ag8.653 mouse myeloma cells. Briefly, spleen cells were fused with myeloma cells using polyethylene glycol (PEG) (Sigma-Aldrich). The fused cells were selected in RPMI 1640 Medium (Gibco; Thermo Fisher Scientific) supplemented with 20% fetal bovine serum (Thermo Fisher Scientific), 2 mM L-glutamine (Gibco; Thermo Fisher Scientific), 50 µM 2-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml of streptomycin, hypoxanthine, aminopterin, and thymidine. Hybrid growth was visually evaluated using an inverted microscope (Olympus IMT-2; Olympus Corporation, Tokyo, Japan). Hybridoma supernatants were collected and subsequently screened for antibodies against hemozoin-associated proteins by slot immunoblotting. Positive hybridoma clones were subcloned to single clones that produced the desired MAbs, after which they were propagated in mini-scale cultures. The MAbs against hemozoin-associated proteins were purified from hybridoma supernatants using Protein G/A Affinity Chromatography (GE Healthcare Life Sciences) as recommended by the manufacturer. The antibody isotypes were identified using a mouse isotyping panel.

All animal procedures were performed according to the protocols approved by the Siriraj Animal Care and Use Committee of the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand (COA no. SI-ACUP 015/2553).

Slot immunoblot assay

The enriched hemozoin-associated proteins were briefly sonicated. The enriched hemozoin (0.3 µg/slot) suspension in PBS or uninfected human hemolysates (0.3 µg/slot) or heme (0.0125 µg/slot) (hemin chloride, H9039; Sigma-Aldrich, St. Louis, MO, USA) was applied to a nitrocellulose membrane (PROTRAN BA-85; Schleicher & Schuell, Dassel, Germany) using a slot blot apparatus (Hybri-Slot Manifold 1052MM; GE Healthcare Life Sciences, Marlborough, MA, USA) under an applied 50 kPa of vacuum. The membrane was incubated in a blocking solution of 5% (w/v) skimmed milk (Bio-Rad Laboratories, Hercules, CA, USA) in PBST for 1 h. The membrane was then incubated with the primary hybridoma supernatants at 1:5 dilutions for 1 h. Following three 5 min washes in PBST, the membrane was incubated for 1 h with a 1: 2,000-diluted alkaline phosphatase-conjugated goat anti-mouse IgGAM antibody (KPL, Gaithersburg, MD, USA). The membrane was then washed with PBST followed by incubation in BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) (KPL, Gaithersburg, MD, USA). Excess substrate was rinsed away with distilled water when the protein bands became visible.

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Western blot analysis

Western blot analysis was performed to evaluate the specificity of the MAbs against hemozoin-associated proteins. The enriched hemozoin preparation was briefly sonicated, applied onto a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel under reducing conditions in total amount 2 μg/lane and transferred to a nitrocellulose membrane for Western blot analysis. The blot was blocked for 2 h in 5% skimmed milk in PBST, followed by incubation for 1 h with primary hybridoma supernatants (1:5 dilution), anti-6xHis antibody (Genscript, Piscataway, NJ, USA) (1: 2,500) or ALP-conjugated Ni-NTA (QIAGEN, Hilden, Germany) (1:500). After washing with PBST, the blot was incubated for 1 h with a 1: 2,000 dilution of ALP-conjugated goat anti-mouse IgGAM antibody, followed by incubation in the BCIP/NBT substrate. The reaction was stopped by rinsing the blot with distilled water.

Immunofluorescence assay (IFA)

Asynchronous cultures of *P. falciparum* isolate NF54-infected erythrocytes were kindly donated by Dr. Jetsumon Sattabongkot of the Faculty of Tropical Medicine, Mahidol University. *P. falciparum* clone 3D7-infected erythrocytes were kindly donated by Prof. Kovit Pattanapanyasat of the Faculty of Medicine Siriraj Hospital, Mahidol University.

For the immunofluorescence assay, blood smears were fixed with prechilled acetone for 5 min and allowed to air dry. The slides were then incubated in a blocking solution of 1% BSA (Sigma-Aldrich) in PBS that contained 10% goat serum for 30 min. After incubation with the appropriate dilutions of the primary antibodies diluted in blocking buffer for 1 h, the slides were then washed in PBS and subsequently incubated for 1 h with 1:20-diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (KPL). Nuclei were stained with Hoechst 33258 dye (Invitrogen, Carlsbad, CA, USA) at a final concentration of 10 μg/ml in blocking buffer. Negative control incubations were performed in the absence of primary antibodies or with an irrelevant antibody or uninfected erythrocytes or mammalian cell line HT-29 (ATCC, Manassas, VA, USA) with primary antibodies. After washing in PBS, the slides were mounted with glycerin and glass coverslips were applied. Slides were visualized using a Nikon Eclipse 80i Fluorescence Microscope (Nikon, Tokyo, Japan) and an Axiovert 200M LSM 510 META Laser Scanning Confocal Microscope (Carl Zeiss GmbH, Jena, Germany). All intraerythrocytic stages of the parasites were investigated, including the ring, trophozoite, and schizont stages. All incubation steps were performed in a humidity box at RT.

Results

Characteristics of hemozoin

The enriched hemozoin preparation was validated using a spectrophotometer (Fig. 1A). That analysis showed a peak with an absorption maximum at 650-665 nm (Fig. 1A, spectrum x, black arrow), which is characteristic of intact hemozoin (Fitch, Kanjananggulpan, 1987). After dissolution of hemozoin

into monomeric heme, the spectrum exhibited a narrow Soret peak at 400 nm (Fig. 1A, spectrum y, black arrow) with an absorption maximum at 600 nm (Fig. 1A, spectrum y, red arrow) (Fitch, Kanjananggulpan, 1987). The hemozoin preparation was also examined using scanning electron microscopy (Fig. 1B). That analysis showed brick-like crystals with approximate dimensions of 100 nmx100 nmx300-500 nm, which is consistent with the structure of hemozoin described in previous studies (Noland et al., 2003). These findings confirmed that the preparation contained hemozoin.
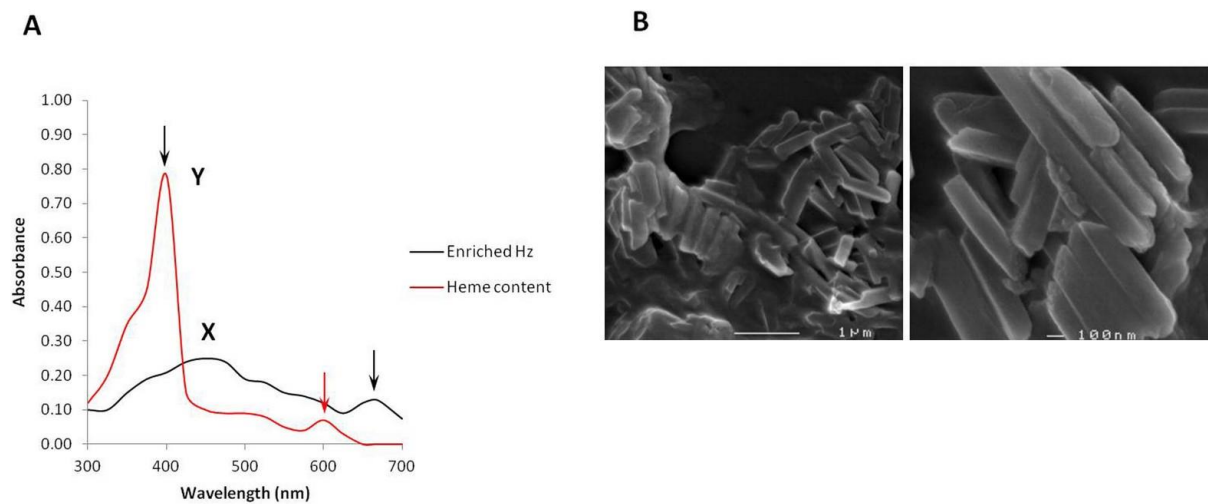
Figure 1   Characteristics of *P. falciparum* hemozoin (Hz). (A) The spectral features of *P. falciparum* hemozoin were measured spectrophotometrically. Spectrum x, suspension of enriched hemozoin in PBS. Spectrum y, dissolution of hemozoin into monomeric heme in 20 mM NaOH/2% SDS. (B) SEM micrographs show the shape and size of enriched *P. falciparum* hemozoin. Scale bars: 1 µm (left), 100 nm (right).

Selection of monoclonal antibodies recognizing hemozoin preparation

The hybridoma supernatants of 6 clones were tested with the enriched hemozoin-associated proteins. Uninfected human hemolysate and heme (hemin chloride) were used as negative controls. Two MAbs, designated 13C7 and 10A2, against hemozoin-associated proteins were selected for the best clones that reacted with hemozoin-associated proteins but no cross reactivity with normal blood hemolysate or heme by slot immunoblotting (Fig. 2A). Western blot analysis revealed that 13C7 MAb recognized a protein band of approximately 40 kDa (Fig. 2B, lane 1). The 10A2 MAb predominantly recognized a 55 kDa band (Fig. 2B, lane 2).
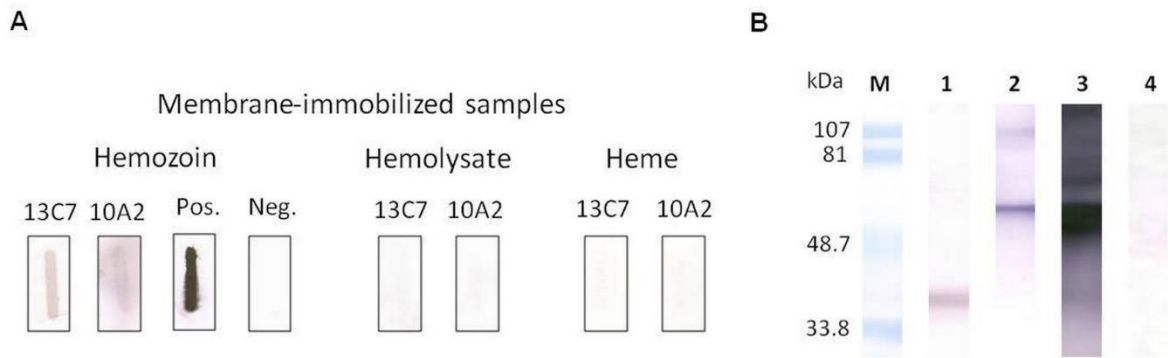
Figure 2   Specific reactivity of the MAbs against hemozoin-associated proteins by immunoblot analysis. (A) Slot immunoblotting of enriched hemozoin preparation was probed with 13C7 and 10A2 supernatants as the primary antibodies. Polyclonal antiserum raised against enriched hemozoin preparation and hybridoma medium were used as positive and negative controls, respectively. The blots of uninfected human hemolysate and heme detected with these MAbs were used as negative controls. (B) Western blotting of enriched hemozoin preparation using 13C7 (lane 1) and 10A2 (lane 2) antibodies. Polyclonal antiserum raised against enriched hemozoin preparation (lane 3) and irrelevant supernatant (lane 4) were used as positive and negative controls, respectively. Lane M, prestained molecular weight marker.

Localization of the MAb binding sites in *P. falciparum*-infected erythrocytes by laser scanning confocal microscopy

Localization of the MAb reactive hemozoin-associated proteins in *P. falciparum*-parasitized erythrocytes was investigated by immunofluorescence staining and visualization by fluorescence and laser scanning confocal microscopy (Fig. 3). Two *P. falciparum* parasites (isolate NF54 and clone 3D7) were used to infect erythrocytes, and the results compared between the two were identical.

The fluorescence staining of the 13C7 MAb was restricted only to the parasite and not observed in any other part of the infected erythrocyte. During ring stage, the fluorescence intensity of 13C7 MAb was clearly observed on the margin surrounding the Hoechst 33258-stained parasite nucleus at the early ring stage, as well as at the late ring stage, during which dark hemozoin is visibly apparent (Fig. 3A). Immunofluorescence was also observed on the small vesicles near the margin surrounding the parasite but not on the outer erythrocyte membrane. The trophozoite stage, the staining intensity increased. The 13C7 MAb primarily exhibited immunofluorescence primarily surrounding the parasite periphery, and on the short tubular or protuberant projections extending from the parasite. Immunofluorescence was also observed on the small vesicles localized near the margin surrounding the parasite. The schizont stage, fluorescence signals were still observed on the margin, with protuberances surrounding the parasites. However, this MAb did not react with the merozoites that were released after the rupture of

the infected erythrocytes and gametocytes. Fluorescence staining was also superimposed on the hemozoin during the trophozoite and schizont stages. The signal that was superimposed on hemozoin was then confirmed and clearly demonstrated by confocal z-stack projections from the top to the bottom of the cell layer (Fig. 3B). This indicated that the structure recognized by the MAb was located close to hemozoin.

In contrast to the 13C7 MAb, fluorescence staining of the 10A2 MAb recognizing the 55 kDa protein was observed in all erythrocytes, *P. falciparum*-infected isolate NF54 or clone 3D7. The fluorescence signal of this MAb was observed diffusely over the entire infected erythrocyte during the ring, trophozoite, and schizont stages (Fig. 3C). During the ring stage, a diffuse pattern of fluorescence in the cytosol of infected erythrocytes was observed. The fluorescence of this MAb was also observed in the internal region of the parasite that surrounded the hemozoin-containing food vacuole. During the trophozoite and schizont stages, two fluorescence signal patterns were observed. The signals were localized primarily at the hemozoin-containing food vacuole and throughout the entire cytoplasm of infected erythrocytes. This MAb also did not react with the merozoites released after the rupture of infected erythrocytes and gametocytes. Fluorescence staining at the sites associated with the hemozoin-containing food vacuole and in the cytoplasm of infected erythrocytes was then confirmed by confocal (Fig. 3D). These data indicated that the 10A2 MAb binding sites were located in the host erythrocyte cytosol and in the hemozoin-containing food vacuole. The staining pattern and the parasite stages associated with this antibody resembled the antibody recognition of the histidine-containing protein (Haeggstrom et al., 2004; Howard et al., 1986; Sullivan et al., 1996).
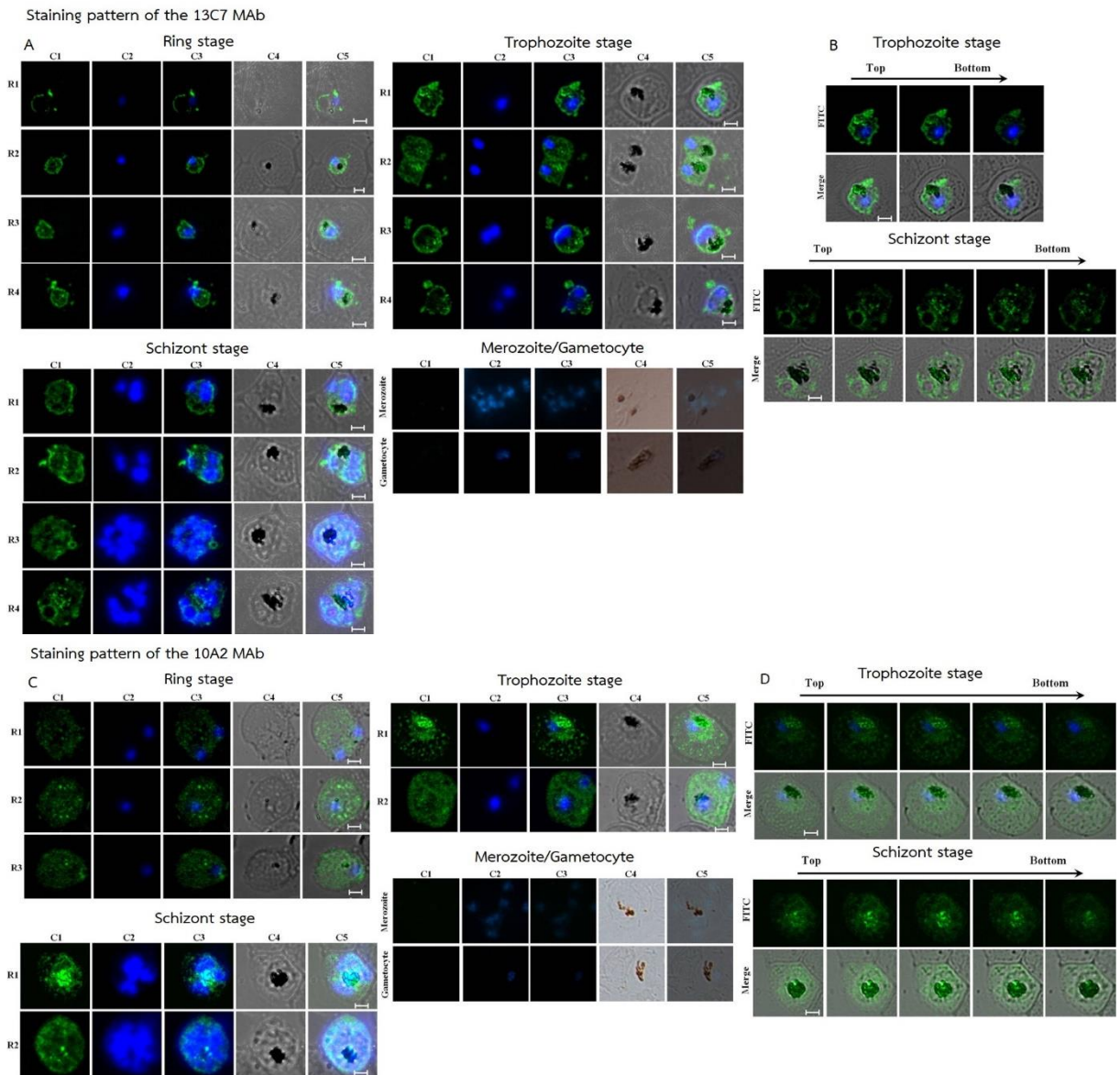
Figure 3 Immunofluorescence reactivity of MAbs against hemozoin-associated proteins with parasitized erythrocytes under fluorescence and laser scanning confocal microscopes. Asynchronous cultures of erythrocytes infected with *P. falciparum* isolate NF54 were reacted with primary 13C7 (panel A-B) or 10A2 (panel C-D) antibodies and then reacted with secondary FITC (green)-conjugated goat anti-mouse IgG antibody (columns 1). Nuclei were stained with Hoechst 33258 dye (blue) (columns 2). Columns 3 are the composites of columns 1 and 2. Columns 4 show the bright field images of the left columns. Merged all images are shown on the right (columns 5). Single projections of z-stacks of about 5 confocal sections (stack z-spacing, 0.29 μm) were shown (panel A, C). For the images (panel B, D) represented the z-stack projection of 5 confocal sections from the top to bottom cell layer, as indicated by the arrow on the top (stack z-spacing, 0.29 μm). Scale bars, 2 μm.

Discussion and Conclusions

Our concept is based on combining the paramagnetic property of hemozoin with the immunologic properties of malarial antigen-antibody interactions. Immunization with hemozoin preparation is not superior in generating antigens compared with conventional methods. Hemozoin is a crystal and is not immunogenic itself. In the present study, we focused on identifying antibodies that react with malarial proteins associated with hemozoin obtained from the magnetic enrichment process.

In this study, the 13C7 MAb recognized a 40 kDa protein associated with *P. falciparum* PVM. Our data indicated that the hemozoin-associated proteins recognized by this MAb was extensively localized on the PVM, and on the tubular/vesicular structures protruding from the PVM during the ring, trophozoite, and schizont stages of the parasite, but not on the merozoites or gametocytes. Several investigators have studied and characterized the parasite-derived proteins that are associated with the PVM. Early transcribed membrane proteins (ETRAMPs) are small PVM-associated proteins. One of which is the 39 kDa ETRAMP10.2, that is expressed and located on the PVM during the blood-stage of the parasite (Spielmann et al., 2003). However, it is also present in merozoites and gametocytes. Exported Protein-2 (EXP-2) is a 35 kDa protein located on the PVM, in membrane-bound compartments as PVM buds into the infected erythrocyte cytosol (Fischer et al., 1998; Johnson et al., 1994), and within the dense granules of merozoites (Bullen et al., 2012). The 13C7 immunofluorescence pattern also resembled EXP-2, in terms of the subcellular localization during the ring, trophozoite, and schizont stages. However, 13C7 MAb did not react with merozoites. These findings suggest that the 40 kDa *P. falciparum* protein recognized by 13C7 MAb in the present study differs from the aforementioned PVM-associated parasitic proteins.

The 10A2 MAb recognized a 55 kDa histidine-containing protein. The best known member among the histidine-containing malarial protein families is PfHRP II. Several studies reported the molecular weight of PfHRP II obtained from extracted infected cells varied from >50 kDa, 69 and 72 kDa (Sullivan et al., 1996). It was much higher than the predicted 29 kDa size based on its amino acid sequence. In this study, the hemozoin-associated proteins recognized by this 10A2 MAb was found to be primarily located in the food vacuoles and in the cytosol of *P. falciparum*-infected erythrocytes during the ring, trophozoite, and schizont stages of the parasite. PfHRP II was initially synthesized at an early stage, it accumulated during parasite growth, and was primarily located in the erythrocyte cytoplasm. The PfHRP II immunofluorescence was observed as diffuse fluorescence over the entire infected erythrocyte, and was distributed as small fluorescent dots when the parasites matured (Howard et al., 1986). Therefore, our immunofluorescence result of 10A2 MAb with both smooth and punctuated pattern resembled this histidine-rich protein.

In this study, we employed the paramagnetic hemozoin to obtain hemozoin-associated proteins for monoclonal antibody production. The established MAbs revealed evidence of hemozoin-associated reactivity by immunofluorescence assay indicating that the proteins recognized by the two MAbs were

present in the hemozoin preparation. Further studies are required to use of immunologic detection by hemozoin-associated protein-antibody interactions combined with the paramagnetic hemozoin to enrich the parasites in infected blood for improving malaria diagnosis in the future.

Acknowledgements

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