Effect of Wheat Germ Agglutinin (WGA) Binding to Red Blood Cell on *Plasmodium falciparum* Invasion

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Red blood cell (RBC) membranes are stable and deformable due to its mechanical properties maintained by the spectrin-based submembranous skeletal network. Therefore, RBC appears not to accept *Plasmodium falciparum* (Pf) invasion easily. However, Pf does invade RBC probably due to change in membrane mechanical properties at the area of Pf contact.

In the present study we examined whether increase in membrane rigidity may prevent Pf invasion into RBC. Wheat germ agglutinin (WGA) was utilized to increase membrane rigidity and double fluorescence assay with a flow cytometer was established; the biotin-WGA-treated RBC (B-WGA-treated RBC) was mixed with Pf-infected RBC followed by staining with streptavidin-FITC and propidium iodide (PI) to detect B-WGA-treated RBC and Pf-infected RBC, respectively.

Having confirmed that membrane rigidity of B-WGA-treated RBC increased significantly using an ektacytometer, we could show that these cells were not infected by Pf using double fluorescence assay. The target molecule of B-WGA was also identified to be band 3 by pull-down assay. These results enabled us to suggest that B-WGA binding to band 3 molecule may induce conformational change in band 3-ankyrin-spectrin, sequentially, resulting in increase in membrane rigidity and thus prevention of Pf invasion into RBC.

Key words : *Plasmodium falciparum*/red blood cell/membrane rigidity/wheat germ agglutinin/band 3

Introduction

Malaria is the most serious disease of human red blood cell (RBC) caused by Protozoan parasite of the genus *Plasmodium* in that *P. falciparum* (Pf) is responsible for the most severe form of malaria¹⁾. About 300-400 million people are infected

per year worldwide with an average of 6,000 (including 3,000 children) deaths per day^{1, 2)}. Sporozoites invade the liver cells and multiplying to thousands followed with release of merozoites. After successful internalization of the merozoite into RBC, asexual division starts and the parasite develop from ring to mature stage and eventually bursts to release merozoite that can reinvade RBC (Fig. 1). Morbidity and mortality occur during the intra-RBC stage of the life cycle of the parasite, and

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Fig. 1 *P. falciparum* (Pf) hepatic and red blood cell (RBC) cycle.

Sporozoites are inoculated to human blood circulation by mosquito (1). The sporozoites invade the liver cells and multiply to thousands (2). Mature schizonts rupture and release merozoites (3), the merozoites invade into RBC, within RBC, the parasites multiply by asexual division, Pf develop through ring, trophozoite and mature schizont stage, respectively (4-7). The mature schizont bursts to release 8-24 merozoites (8), which reinvade new RBC or merozoites may differentiate into sexual RBC stages (gametocytes).

it is this stage that most scientists are investigating for drug and vaccine developments.

RBC membrane is stable and reasonably rigid due to its mechanical properties maintained by the spectrin-based submembranous skeletal network. Therefore, RBC appears not to accept Pf invasion easily. However, Pf does invade RBC probably due to change in membrane mechanical properties at the area of Pf contact⁽³⁾. It is thus reasonable to assume that upon Pf contact with RBC, membrane rigidity may locally decrease due to signal transduction through conformational changes of trans-membrane protein-anchor protein-membrane skeletal proteins, such as spectrin. In the present study we examined whether increase in membrane rigidity prevent Pf invasion into RBC. For this purpose wheat germ agglutinin (WGA) was utilized since this lectin has been shown to increase rigidity of RBC membranes⁽⁴⁾. In order to quantify para-

sitemia (number of Pf-infected RBC) we used our newly developed double fluorescence assay with a flow cytometer. The biotin-WGA-treated RBC (B-WGA-treated RBC) was mixed with Pf-infected RBC followed by staining with streptavidin-FITC to detect B-WGA-treated RBC. The propidium iodide (PI) which specifically binds to DNA was simultaneously used to detect DNA in Pf-infected RBC.

Having confirmed that membrane rigidity of B-WGA-treated RBC increased significantly using an ektacytometer, we could show that these cells were not infected by Pf using double fluorescence assay as described above. The target molecule of B-WGA was also identified to be band 3 by pull-down assay. These results enabled us to suggest that B-WGA binding to band 3 molecule may induce conformational change in band 3-ankyrin-spectrin, sequentially, resulting in increase in

membrane rigidity and prevention of Pf invasion.

Materials and Methods

Materials

Human venous blood was obtained from healthy volunteers after obtaining informed consent. Blood was mixed with anticoagulant-Citrate Phosphate Dextrose (CPD) and stored at 4°C. The blood was used within three days after collection. For Pf culture, whole blood was mixed with anticoagulant-Acid Citrate Dextrose (ACD) and left at 4°C for at least 5 days or fresh blood was used after removal of leukocytes using Terumo kit III-RC (TERUMO, Japan). For preparation of non-immune serum, blood from group O (+) donor was left to clot and the serum was collected and inactivated at 56°C for 30 min. Biotin-wheat germ agglutinin (B-WGA), Streptavidin-FITC were from VECTOR Laboratories (Canada) and Wako (Tokyo, Japan) respectively. Polyvinylpyrrolidone (PVP) K 90 and Propidium iodide (PI) were obtained from Nacalai tesque Inc. (Kyoto, Japan). Monoclonal anti-glycophorin A (GPA) and glycophosin C (GPC) antibodies were obtained from Sigma-Aldrich (USA). Streptavidin Sepharose, Anti-Rabbit Immunoglobulins conjugated to Horse Radish Peroxidase were purchased from Amersham Pharmacia Biotech. (UK) and (DAKO A/S, Denmark), respectively. Polyclonal anti-band 3 antibody was produced in our laboratory. RPMI 1640 Medium powder with L-glutamine, 7.5% (v/w) NaHCO₃, and Hepes were ordered from GIBCO (USA). Chloroquine-sensitive strain *P. falciparum* F32 was obtained from Karolinska Institute (Sweden).

Measurement of RBC membrane rigidity

Twenty μ l of washed RBCs were mixed with 4 ml of 3.5% PVP in PBS containing 0, 0.5, 1.0, 2.5

and 5.0 μ g/ml of B-WGA, followed by incubation at room temperature with gentle mixing. Membrane rigidity was measured by an ektacytometer as described previously⁵⁾. Briefly, RBCs were subjected to linearly increasing shear stress up to 150 dynes/cm², and change in laser diffraction patterns from a circle to an ellipse was recorded as a deformability index (DI)⁶⁾. DI provides a measure of ellipticity of deforming RBC (namely spectrin extensibility) in the rotational flow field⁵⁾. DI was plotted as a function of applied shear stress. DI increase curve indicates RBC membrane rigidity and thus the slower increase reflects higher rigidity.

Treatment of RBC with B-WGA for *in vitro* Pf invasion assay

B-WGA (0 and 5 μ g/ml) in PBS were mixed with human RBC of group O (+) followed by incubation at room temperature for 20 min with gentle mixing. The mixture was then washed three times in PBS to remove unbound B-WGA. At these concentrations neither cell aggregation nor haemolysis was observed. The RBC pellets with or without B-WGA were used for *in vitro* Pf invasion assay. Blood of group O (+) was used generally for Pf culture because it is compatible with serum or plasma of all other blood groups⁷⁾.

Preparation of Pf-infected RBC for *in vitro* Pf invasion assay

Pf was grown in continuous culture⁸⁾ with RPMI 1640 medium supplemented with 10% heat-inactivated O (+) non-immune human serum, 25mM HEPES, 25mg/l gentamycin sulfate and 24mM NaHCO₃ and a 2% suspension of human RBC of group O (+) at 37°C under gas conditions of 7.5% O₂, 5% CO₂ and 87.5% N₂.

Stages of Pf were synchronized with sorbitol⁹⁾ followed by continuous culture to obtain mature

stage parasites such as late trophozoite and schizonts. Sorbitol was used to eliminate chemical or temperature stress that may affect the invasive stage of Pf. RBCs containing sorbitol-synchronized mature parasites, hereafter referred to as an inoculum, was used throughout our experiments. Percentage of parasitemia was calculated to be about 10% by counting the number of infected RBC in 1,000 RBC with Giemsa staining under light microscopy. It should be pointed out that the inoculum contained not only mature Pf-infected RBC (about 10%) but also a significant number of Pf-uninfected intact RBC (about 90%).

***In vitro* Pf invasion assay**

An inoculum (containing Pf-infected and -uninfected RBCs) was mixed with B-WGA-treated or untreated RBCs in complete medium RPMI 1640 at a ratio of 1 to 18. One ml of the mixture was dispensed to each of the micro well culture plate in triplicate followed by incubation at 37°C with 7.5% O₂, 5% CO₂ and 87.5% N₂ for 48 hr.

For flow cytometric analysis, double fluorescence of streptavidin-FITC and PI were used to detect B-WGA-treated RBC and DNA of Pf-infected RBC, respectively. RBC were harvested, washed and re-suspended in PBS. Streptavidin-FITC was added and incubated at room temperature for 30 min in the dark. After washing, RBC in the pellet was fixed and permeabilized as described¹⁰⁾ with slight modification. Briefly, RBC was suspended in 0.025% glutaraldehyde in PBS to a haematocrit of 2% and incubated at room temperature for 20 min. After washing, RBC was permeabilized with 1% (v/w) saponin in PBS containing 1% BSA, followed by incubation at room temperature for 15 min. After washing, 10 µg/ml of PI was added and left at room temperature for 20 min in the dark.

Resolution was checked for each sample by using fluorescence microscope (Eclipse E600, Nikon, Japan) before proceeding to flow cytometric analysis. Sample was applied and quantified by the flow cytometer (EPICS-XL, Beckman Coulter Co.).

Pull-down assay

RBCs (100 µl) were suspended in 4 ml PBS containing B-WGA (4 µg/ml) and incubated for 30 min at room temperature. Cells were collected, washed with PBS, and suspended in PBS containing 1% (v/v) Triton X-100. After incubation on ice for 30 min, the suspension was centrifuged at 6,000 × g for 10 min at 4°C. The supernatant (TX-sup) was collected and the pellets re-suspended in 0.1 mM EDTA (pH 8.3) followed by incubation at 37°C for 1 hr. Following centrifugation (100,000 × g, 15 min) collected supernatant was diluted with PBS (EDTA-sup). Streptavidin-Sepharose was added to these supernatants (TX-sup and EDTA-sup) followed by incubation at 4°C for 2 hr or overnight with gentle mixing. The Sepharose beads were collected by sedimentation, washed by PBS, and re-suspended in the washing buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (w/v) Nonidet P-40, 0.1% (w/v) SDS, and 0.5% (w/v) sodium deoxycholate). After incubation at room temperature for 10 min with gentle mixing the beads were collected and further incubated in 10 mM phosphate buffer (pH 7.4) containing 1 M NaCl for 10 min. Pulled-down proteins were denatured in SDS-sample buffer (125 mM Tris-HCl (pH 6.8), 4.6% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, and 20 µg/ml Bromophenol Blue) by heating at 100°C for 5 min. The samples were then analyzed by 9% SDS-PAGE followed by silver stain and immunoblotting¹¹⁾.

Fig. 2 Measurement of RBC membrane rigidity using an ektacytometer.

DI of the control RBC (line a) and B-WGA-treated RBC (lines b-e) were monitored with increasing shear stress up to 150 dyn/cm². DI for the control RBC increased rapidly to reach DI_{max} of 0.8 (line a) whereas B-WGA-treated RBC showed a slower rate of DI increase dependent on B-WGA concentrations of 0.5 (line b), 1.0 (line c), 2.5 (line d) and 5.0 (line e) mg/ml, respectively.

Results

Increase in RBC membrane rigidity by B-WGA

Membrane rigidity of B-WGA-treated RBC were measured by an ektacytometer (Fig. 2). When the control RBCs were subjected to linearly increasing applied shear stress up to 150 dyn/cm², the deformability index (DI) increased, reaching a maximum DI level (DI_{max}) of 0.8 (line a). B-WGA-treated RBCs showed a slow DI increase curve, indicating that the membrane rigidity was increased by B-WGA treatment. This effect was enhanced with increase in B-WGA concentrations up to 5 µg/ml (lines b-e). Since shape of B-WGA-treated RBC still showed normal biconcave disc form (data not shown), DI increase corresponds

mainly to the extensibility of spectrin. If that is the case, B-WGA binding on the RBC surface may induce decrease in spectrin extensibility in the sub-membranous membrane skeleton through some transmembrane signal transduction. It should be noted that neither cell aggregation nor haemolysis was observed at B-WGA concentrations up to 5 µg/ml.

Flow cytometric measurement of Pf infection

We examined whether Pf can invade into B-WGA-treated RBC with extremely rigid membranes by a flow cytometric measurement as described in Materials and Methods. Two fluorescent dyes such as streptavidin-FITC and PI were used to detect B-WGA-treated RBC and Pf-infected RBC, respectively. Simultaneous detection using FITC and PI enabled us to observe separately 4 populations in each window 1 to 4 (see figure legend for Fig. 3A).

Using Pf-uninfected RBC with or without B-WGA treatment, we firstly demonstrated that these two RBC populations could be observed separately by their differences in FITC fluorescence intensity. As shown in Fig. 3B, following mixing equal numbers of RBC with low FITC intensity and B-WGA-treated RBC with high FITC intensity, almost equal distribution was observed in windows 1 and 2.

Using WGA-untreated RBC, we then made sure that Pf-infected RBC were detected separately from Pf-uninfected RBC by their differences in PI fluorescence intensity. As shown in Fig. 3C, following 48 hrs incubation of the inoculum (containing 10% Pf-infected and 90% -uninfected RBC) with RBC at a ratio of 1:18, the flow cytometric analysis of this sample showed that 4.4% of Pf-newly infected RBC with high PI intensity was observed in window 3 and 93.4% of Pf-uninfected RBC with low PI intensity in window 1. It should be noted that original Pf-infected RBC (10%) were ruptured during incubation.

Fig. 3 Flow cytometric analysis of Pf infection into B-WGA treated RBC.

RBCs were analyzed by a flow cytometer (EPIC-XL, Beckman Coulter Co.) as described in Materials and Method. Analysis windows were set up for FITC on the X-axis and PI on the Y-axis. Fig. 3A will show definitions for each window; Window 1 corresponds to the control RBC that is B-WGA-untreated and Pf-uninfected RBC. Window 2 represents B-WGA-treated but Pf-uninfected RBC. In window 3 there are B-WGA-untreated but Pf-infected RBC. Window 4 represents WGA-treated and Pf-infected RBC that is stained with both FITC and PI. Fig. 3B shows distribution of the mixture of B-WGA-treated and -untreated RBC at a ratio of 1 to 1. Fig. 3C represents distribution of the mixture of Pf-infected and -uninfected RBC at a ratio of 1 to 18 after 48 hrs incubation. Fig. 3D shows distribution of the mixture of Pf-infected RBC and B-WGA-treated RBC at a ratio of 1 to 18 after 48 hrs incubation. It should be noted that Pf-infected RBC sample (inoculum) contains 10% Pf-infected RBC and 90% Pf-uninfected RBC. The number of Pf even after RBC rupture is much less than the total number of RBC in Figs. 3C and 3D.

tion, releasing an average of 10 Pf per RBC. These Pf infected both RBCs which were contained in the inoculum (containing 90% Pf-uninfected RBC)

and newly added RBC that is 18 times the number of the inoculum.

Having established the flow cytometric analysis

Fig. 4 Identification of the B-WGA-binding protein on RBC.

B-WGA was pulled-down from TX-sup and EDTA-sup by streptavidin-Sepharose as described for Material and Methods. Proteins pulled-down with B-WGA from TX-sup (lanes 2 and 3) and EDTA-sup (lanes 4 and 5) were analyzed by SDS-PAGE (9%). The supernatants were prepared from B-WGA-untreated (lanes 2 and 4) or -treated (lanes 3 and 5) RBC. Panel A: silver stain. Panel B-D : immunoblotting using anti-band 3 (B), anti-GPA (C), and anti-GPC (D) antibodies. Ghost was used as standard for RBC membrane proteins (lane 1).

technique we examined whether Pf invaded into B-WGA-treated RBC. As shown in Fig. 3D, following 48 hrs incubation of Pf-infected RBC sample with B-WGA-treated RBC at a ratio of 1:18, the flow cytometric analysis of this sample showed that 3.6% of Pf-newly infected RBC with high PI intensity was observed in window 3 and 95.8% of Pf-uninfected RBC with high FITC intensity was seen in window 2. The fact that no RBC was observed in window 4 indicates that Pf did not invade into B-WGA-treated RBC. It should be noted that 3.6% of Pf-newly infected RBC in window 3 were derived from about 90% of Pf-uninfected RBC which were contained in the inoculum.

Identification of the B-WGA-binding protein on RBC

To identify B-WGA-binding proteins on RBC sur-

face, we used pull-down assay with streptavidin-Sepharose as described in Materials and Methods. Since membrane skeletal proteins were not easily solubilized by 1% (v/v) Triton-X100 (due to tight associations to each other), two-step solubilization was performed as described for Materials and Methods. Solubilized B-WGA was pulled-down by streptavidin-Sepharose and proteins simultaneously sedimented were analyzed by SDS-PAGE and immunoblotting. As shown in Fig. 4A, band 3 was detected in TX-sup (lane 3) as well as EDTA-sup (lane 5). Presence of band 3 was confirmed by immunoblotting with an anti-band 3 antibody (Fig. 4B, lanes 3 and 5). These results indicate that under the present experimental conditions B-WGA appears to bind to sugar chains of band 3 protein. We then examined whether GPA and GPC were also recognized by B-WGA since these gly-

cophorins with several sugar chains are considered to be a possible binding molecule of WGA. GPA was detected as a dimer and a monomer on RBC ghost (Fig. 4C, lane 1) while GPA was not detected in pulled-down fraction in TX-sup and EDTA-sup (Fig. 4C, lanes 3 and 5). GPC present as a monomer on RBC ghost (Fig. 4D, lane 1) was not detected in both pulled-down fractions (Fig. 4D, lanes 3 and 5). These results indicate that band 3, but not GPA and GPC, is at least one of the B-WGA-binding protein on RBC.

Discussion

In the present study we could show that increase in membrane rigidity prevented invasion of Pf into RBC. B-WGA was used to increase in membrane rigidity. To quantitate parasitemia a flow cytometer was utilized for double fluorescence assay with streptavidin-FITC and PI to detect B-WGA-treated RBC and Pf-infected RBC, respectively.

We first confirmed that B-WGA (as well as WGA) increased RBC membrane rigidity using an ektacytometer. This is consistent with the previous report showing WGA-induced increase in RBC membrane rigidity⁴⁾. Since the shape of B-WGA-treated RBC still showed normal biconcave disc form, the observed increase in rigidity may not be derived from loss of effective surface area⁴⁾. Although WGA at high concentrations may aggregate RBCs, neither cell aggregation nor haemolysis was observed at B-WGA concentrations we used in the present study. These results enabled us to use B-WGA-treated RBC as RBC with significantly increase in membrane rigidity.

Membrane rigidity is maintained by the spectrin-based membrane skeleton which underlies RBC membranes. Especially increase and decrease in extensibility of spectrin decrease and increase in membrane rigidity, respectively. The membrane

skeleton interacts with transmembrane proteins such as band 3 and GPC through anchor proteins of ankyrin and protein 4.1, respectively¹²⁾. In the present experiments, band 3 was identified as the only target for B-WGA using a pull-down assay (Fig. 4), indicating that the signal of B-WGA binding to band 3 reached spectrin molecule through conformational change of band 3-ankyrin-spectrin sequentially and the extensibility of spectrin might decrease with an increase in rigidity.

Having confirmed the effect of B-WGA we examined whether an increase in RBC membrane rigidity may prevent Pf invasion into RBC using B-WGA-treated RBC. We established a flow cytometric analysis with double fluorescences, streptavidin-FITC and PI, to quantitatively measure parasitemia of B-WGA-treated and -untreated RBC. With this technique we have been able to separate RBC into four distinct populations with combinations of FITC (+/-) and PI (+/-) (Fig. 3). The results clearly showed that there was no detectable double positive RBC (Fig. 3D), indicating that B-WGA treatment prevented Pf invasion into RBC.

Mechanism for WGA-induced prevention of Pf invasion is most likely due to increase in RBC membrane rigidity. RBC membranes with increased rigidity must not be easily invaginated following Pf contact. However, we cannot completely exclude the (possible) competition for RBC receptor between WGA and Pf-derived factors, such as MSP1¹³⁾ and EBA-175^{14, 15)} that are necessary for Pf invasion. Since MSP1 has been shown to bind band 3, B-WGA may compete with MSP1 for band 3 on the RBC surface. Furthermore, GPA has been reported as a RBC receptor for EBA-175^{14, 16)} and target for WGA^{4, 17)}. Therefore, one might suggest that WGA competes with EBA-175 for GPA on the RBC surface, resulting in prevention of Pf invasion. However, in our hands band 3 (not GPA) was only the target of B-WGA

(Fig. 4). It is, therefore, unlikely for B-WGA to interrupt binding of EBA-175 to GPA.

In the present experiments the increase in rigidity by WGA is equally distributed through the entire RBC membranes and as such Pf poorly invaded. Although WGA cannot be used *in vivo* as anti-parasite invasion drug, some harmless factor affecting membrane mechanical properties may be available as a drug for prevention of Pf invasion in future. Moreover, present results enable us to assume that during initial stage of Pf invasion the RBC membrane rigidity is locally decreased, allowing invagination upon Pf contact. Identification of Pf-derived factors which induces decrease in membrane rigidity is required to understand precisely the molecular mechanism for Pf invasion into RBC.

Abbreviations : Pf, *Plasmodium falciparum*; RBC, red blood cell; B-WGA, biotin-wheat germ agglutinin; GPA, glycophorin A; GPC, glycophorin C; FITC, fluorescein isothiocyanate

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