Original Contribution : MEMBRANE, 32 (2), 95-102 (2007)

Erythrocyte Shape Change Prevents *Plasmodium falciparum* Invasion

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Normal erythrocytes have biconcave discoid shape that presents large surface area with higher cell surface to volume ratio than that of spherical shape. This appears to allow membrane internalization required for *Plasmodium falciparum* (Pf) invasion into erythrocytes. Indeed abnormal erythrocyte shape with decreased surface area to volume ratio such as hereditary spherocytosis limits invasion of the parasite. In the present study, using several agents to induce erythrocyte shape changes, we examined whether echinocytic shape change with membrane projections in opposite direction to membrane internalizations and/or stomatocytic shape change with decreased surface area to volume ratio that would be required for internalization, prevent Pf invasion. Having microscopically confirmed echinocytic and/or stomatocytic shape changes and also measured extensibility using an ektacytometer of the treated cells, subsequent Pf invasion assay was performed and parasitaemia determined. Both sodium flouride (NaF) and phospholipase A_2 (PLA_2) induced echinocytic change whereas phospholipase D (PLD), sphingomyelinase (SMase) and chlorpromazine (CPZ) caused stomatocytic change with decreased extensibility of erythrocytes. In both situations, Pf invasion was prevented, indicating that biconcave discoid shape of normal erythrocytes with high surface to volume ratio is required for membrane internalization when Pf invades into erythrocytes.

Key words : *Plasmodium falciparum* / erythrocyte / cell surface to volume ratio / cell shape change / membrane extensibility

1. Introduction

Clinical malaria cases estimated as 300-500 million per year[1] could mainly be attributed to *Plasmodium falciparum* (Pf) which has a wider geographical distribution. The erythrocytes are the target of Pf invasion, enlargement and multiplication. The rupture of the schizonts to release merozoites begins another cycle of erythrocytic stage and also marks the onset of clinical malaria episodes[2]. The Pf erythrocytic cycle occurs when the merozoite collides with an erythrocyte where initial interactions ensue. The merozoite randomly attaches, and rapidly brings the apical end in apposition to the surface of erythrocyte, and its entry is accompanied with membrane internalization (invagination) [3, 4]. The invasion process though rapid, is influenced by factors such as surface molecules of the erythrocytes[5, 6], rigidity of the membrane[7], and the shape[8]. These factors confer resistance to malaria infection when they are inherited, which manifest as integral membrane defects mainly ovalocytosis, elliptocytosis and spherocytosis, and enzyme deficiency as in glucose-6-phosphate dehydrogenase deficiency with its resultant basket erythrocyte shape[2, 9, 10].

Normal erythrocytes have biconcave discoid shape that is an important signpost for the functional properties of the cell. The morphology is determined largely by the organization of the plasmalemma composed of the lipid bilayer and proteins including transmembrane and membrane skeletal proteins. Pf invasion into erythrocytes is a prelude to parasite growth and multiplication, all of which depend mainly on the function of

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the erythrocyte membrane by changing its elasticity, permeability and opening nutrient uptake channels[11]. Alteration of a unique membrane property; extensibility, primarily determined by cell surface area to volume ratio (effective surface area) and intrinsic membrane rigidity[7, 12] are likely to interfere with merozoite invasion.

It is generally accepted that the biconcave discoid shape of normal erythrocytes and the inducible shape alterations are primarily influenced by the lipid distribution in the membrane bilayer[13]. The echinocytogenic and stomatocytogenic agents cause erythrocyte shape changes through different mechanisms. Though contentious, the bilayer couple model[14 ~ 16] is a generally accepted mechanism for erythrocyte shape changes and was proved by changing the ratio of lipids and lipophilic agents of the two monolayers[17]. However, under physiological conditions, transbilayer phospholipid movements are too slow to explain shape changes that occur in a matter of seconds[18]. Sodium flouride (NaF) for instance, depletes ATP needed by aminophospholipid translocase (flippase) to transport aminophospholipids mainly phosphatidylserine (PS) from the outer to inner leaflet, thereby causing the expansion of outer layer with echinocytic change[19]. Chlorpromazine (CPZ) induces stomatocytic change by interacting with PS in the inner leaflet of the membrane causing the expansion of inner layer and reducing surface area[14].

The realization of the important role played by erythrocyte membrane in the Pf invasion process prompted intense investigation to unravel the molecular mechanisms that either facilitate or inhibit the process. We therefore tested the hypothesis that erythrocyte large effective surface area is required for efficient Pf invasion. To achieve this, echinocytogenic and stomatocytogenic agents were used to manipulate the erythrocyte shape in order to reduce effective surface area that can be used for internalization. It was observed in this study that both NaF and phospholipase A_2 (PLA_2) induced echinocytic change whereas phospholipase D (PLD), sphingomyelinase (SMase) and CPZ caused stomatocytic change with decreased extensibility of erythrocytes. In both situations, Pf invasion was prevented, indicating that biconcave discoid shape of normal erythrocytes with high surface to volume ratio is required for membrane internalization when Pf invades erythrocytes.

2. Materials and Methods

2.1 Materials

N[2-hydroxyethyl]piperazine-N-[2-ethanesulphonic acid] (HEPES) was obtained from Sigma-Aldrich. RMPI 1640 medium powder supplemented with L-glutamine, sodium bicarbonate solution (7.5% (v/w) and gentamycin solution (50 mg/ml) were obtained from Gibco (USA). In addition, Chlorpromazine [CPZ] (Wako, Japan), Sodium Fluoride [NaF] (Wako, Japan), Phospholipase A_2 [PLA_2] (Worthington Biochemical Corporation, USA), Phospholipase D [PLD] (Biomol International, USA), and Sphingomyelinase [SMase] (Biomol International, USA) were all obtained in highest grade available. Human blood type O^+ was collected from informed and consented healthy malaria non-immune individuals (adults). Acid Citrate Dextrose (ACD) collected blood was filtered to remove leucocytes using Polyurethane filter Kit (Terumo). Erythrocytes were kept at 4 ℃ for at most 10 days or used in fresh form. Blood group O^+ serum was obtained when collected non-immune blood was allowed to clot, centrifuged and, the supernatant inactivated at 56 ℃ for 30 minutes and filtered when cooled. Chloroquine sensitive *Plasmodium falciparum* (Pf) strain (F32) was obtained from Karolinska Institute (Sweden)

2.2 Methods

2.2.1 Treatment of human erythrocytes with reagents

Venous blood drawn from healthy adult volunteers was ACD anticoagulated, filtered and; centrifuged at 3,000 × g for 5 minutes to remove the plasma and the buffy coat. The erythrocytes in the pellet were washed three times with 0.1% glucose phosphate buffered saline (145 mM NaCl, pH 7.4, 0.1% glucose; PBS). Erythorocytes (50 μl) were added to 500 μl of PBS without or containing a specific concentration of either the agents (NaF, CPZ) or enzymes (PLA_2, PLD and SMase) and incubated at 37 ℃ for 1 hr. After washing (1x) with PBS, some of the treated cells were examined microscopically and also used for Pf invasion assay.

2.2.2 Observation of shape change

The morphology of the unfixed, treated and untreat-

ed human erythrocyte was observed by using dark field light Microscopy (Nikon, Optiphot-2) as described[19].

2.2.3 *In vitro* Pf culture

Erythrocytic stages of *Pf* (CHQ sensitive strain F32) were cultured in flasks (75 cm^2 Nunc) using a method of Trager and Jensen[20] with modifications. Erythrocytes were maintained at 4% haematocrit (v/v) cell suspension in complete medium (RPMI 1640, buffered with 25 mM HEPES, 24 mM $NaHCO_3$, supplemented with 25 μg/ml gentamycin and 10% heat-inactivated human $O^{(+)}$ serum) incubated at 37 ℃ under gas conditions of 7.5% O_2, 5% CO_2 and 87.5% N_2. Parasite growth and development were monitored with Giemsa stained thin blood smeared. Pf culture was synchronized with sorbitol[20, 21] and further cultured until matured erythrocytic stages (late trophozoites and schizonts) were obtained.

2.2.4 Pf invasion assay

Sorbitol synchronized but matured erythrocytic stages Pf culture were gradient separated from uninfected cells[22]. The matured stages obtained were added to drug (NaF, CPZ) or enzyme (PLA_2, PLD and SMase) treated erythrocytes in 48 wells plate (Nunc) incubated with complete malaria medium until it is harvested after 20 hrs. Slides were then prepared, Giemsa stained and parasitaemia determined by counting the number of infected cells in a total of 3,000 cells. Briefly the procedure is outlined as; treated erythrocytes (4.4 μl) were seeded in the 48-well plate (Nunc) to which 220 μl complete malaria culture medium (MCM) and 6 μl of isolated matured (schizonts) Pf were added, and incubated at 37 ℃ under gas conditions as stated above. In all cases, untreated erythrocytes were used as control. The Pf invasion assay was premised on the assumption that the schizonts added to the treated cells would rupture and the released merozoites would invade the cells within 1 hr of simultaneous addition and incubation. However, the 20 hrs continuous culture was to ensure the growth of the invaded merozoites to be detected by microscope.

2.2.5 Measurement of membrane deformability and extensibility

Erythrocytes treated with either echinocytogenic or stomatocytogenic agents were assessed for their membrane deformability using an ektacytometer as described[19]. Briefly, each treatment was re-suspended in a stractan (viscosity of 22 centipose, 290mOsm) and examined by an ektacytometer when the cells were subjected to linearly increasing shear stress (0-150 dynes/cm^2), and changes in laser diffraction patterns were analyzed to derive the deformability index (DI). The rate of increase in DI is a measure of membrane deformability, and the maximal value (DI_{max}) depends on the surface area of the treated cells related to membrane extensibility. In all cases, untreated cells were used as control.

3. Results

3.1 Erythrocyte morphology changes induced by agents

To investigate the effect of cell shape change on Pf invasion, erythrocytes were treated with shape changing agents (echinocytogenic and stomatocytogenic) at varying concentrations. Erythrocytes suspended in each agent were incubated at 37 ℃ for 1 hr, rinsed and the morphology examined microscopically. Fig. 1 (B-F) clearly demonstrates the morphology of each representative agent. Fig. 1 B and C were echinocytic induced by NaF and PLA_2, respectively, whereas D, E and F represent stomatocytic change induced by PLD, SMase and CPZ, respectively, when compared with control (Fig. 1A). As observed with each agent, progressive changes in erythrocytes shape were a function of concentration (data not shown). Dramatic shape changes were observed at the maximum concentration NaF (1.7×10^{-1}M), PLA_2 (72.7×10^{-3} μg/μl), PLD (3.6×10^{-3} μg/μl), SMase (1.7×10^{-3} μg/μl) and CPZ (100 μM); consistent with varying mechanism of action of each agent[15, 16, 19, 23]. However, no uniform shape change was observed with increasing concentrations for each treatment since discocytes still remained. The phospholipases and the SMase used were provided in their highly purified grades which were expected to degrade the phospholipids located within the outer leaflet hence no conspicuous lysis observed with each treatment though the shapes of the cells were altered.

3.2 Effects of erythrocyte shape change on Pf invasion

Both echinocytes and stomatocytes caused by the

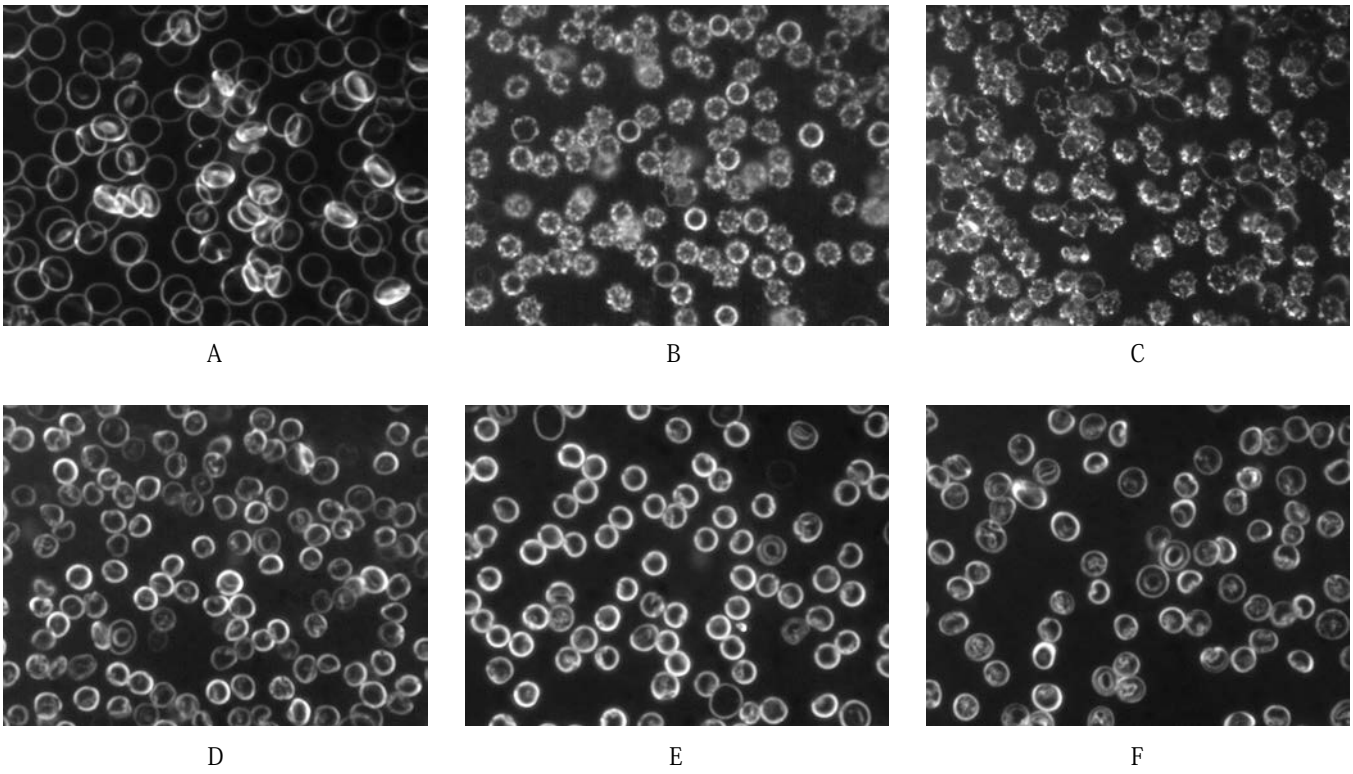
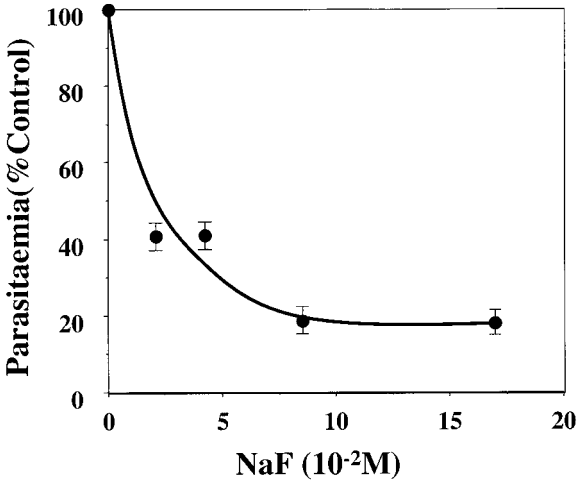


Fig. 1 Dark field micrographs showing morphologic transformations of erythrocytes after treatment with either echinocytogenic or stomatocytogenic agents; A: Untreated cells suspended in PBS at 37 ℃ for 1 hr, rinsed and incubated with MCM for 1 hr. B-F Micrographs were obtained from respective agents at the maximum concentration(s) in which erythrocytes were suspended at 37 ℃ for 1 hr, rinsed and incubated with MCM for 1 hr. B: NaF (1.7×10^{-1}M), C: PLA_2 (72.7×10^{-3} μg / μl), D: PLD (3.6×10^{-3} μg/ μl), E: SMase (1.7×10^{-3} μg/ μl) and F: CPZ (100 μM) Whereas NaF and PLA_2 treated cells were echinocytic, the PLD, SMase and CPZ treated cells were all stomatocytic.

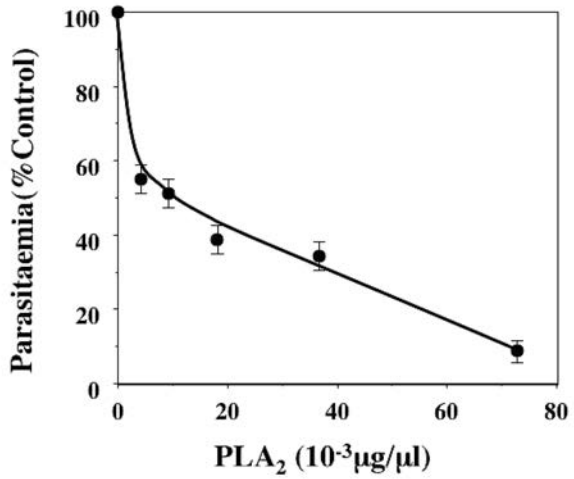
agents used in this study prevented Pf invasion as demonstrated by Fig. 2 (A-E). Generally the pattern of prevention (Fig. 2 A-E) of Pf invasion of treated erythrocytes was concentration dependent, though the qualitative degrees of erythrocyte shape change as induced by the agents vary. However, it is consistent with the progressive shape change as function of concentration. Nonetheless, except Fig. 2 B and E, the rest could not probably have achieved complete prevention even if the maximum concentrations were increased. Compared with the control the concentrations of the agents that inhibited 50% of the Pf invasion were 4.25×10^{-2}M NaF, 9.1×10^{-3} μg/ μl PLA_2, 4.5×10^{-4} μg/ μl PLD, 2.1×10^{-4} μg/ μl SMase and 20 μM CPZ. It is incongruous to make conclusive comparison because each agent induced erythrocyte shape change by different mechanism.

3.3 Effects of echinocytogenic and stomatocytogenic agents on erythrocyte membrane deformability.

The effect of each agent on membrane mechanical properties was assessed by ektacytometry (Fig. 3). When the control erythrocytes were subjected to linearly increasing applied shear stress up to 150 dyn/cm^2, the deformability index (DI) increased reaching a maximum DI level (DI_{max}) of 0.8 (line, a). The findings suggest that membranes of NaF and PLA_2 treated cells, and that of control are indistinguishable in terms of their membrane deformability but the surface area of PLA_2 treated cells slightly decreased compared with that of NaF and control. Conversely membranes of CPZ, PLD and SMase treated cells showed decreased DI value as well as DI_{max} in decreasing order of PLD, SMase and CPZ. Decreased DI_{max} corresponds mainly to reduced extensibility of spectrin. It implies that the expansion of the inner leaflet of the membrane affects spectrin and also results in decreased cell surface area to volume ratio.



A



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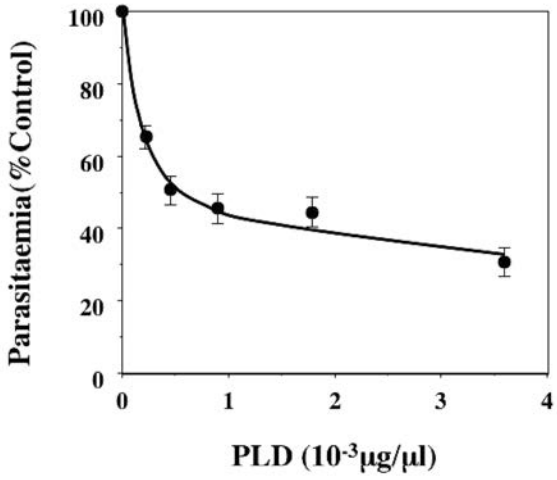

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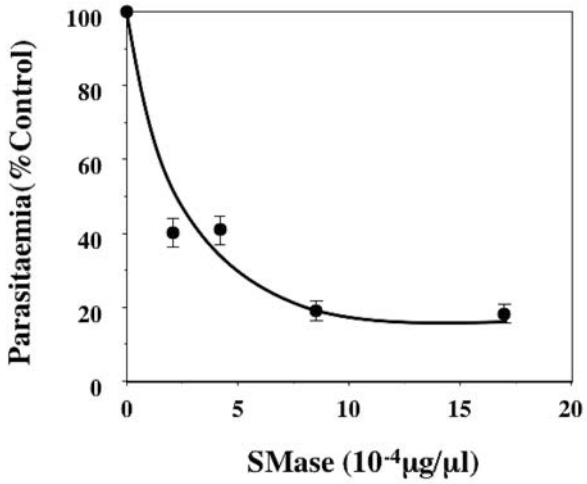
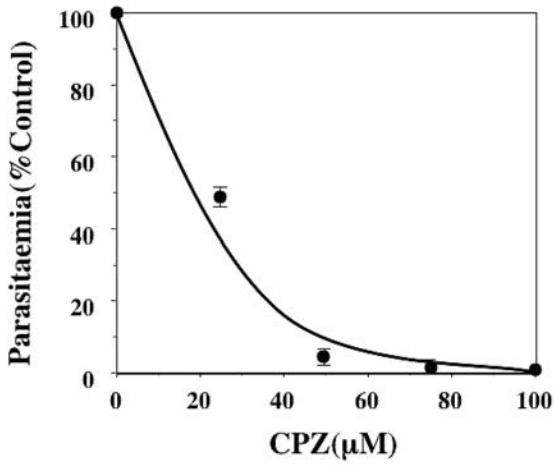


D



E

Fig. 2 Concentration dependent prevention of Pf invasion into erythrocytes treated with either echinocytogenic or stomatocytogenic agents; NaF (A), PLA_2 (B), PLD (C), SMase (D) and CPZ (E). Erythrocytes were suspended in varying concentrations of each of the agents above, rinsed and cultured with purified schizonts for 20 hrs. The cultures were harvested, examined microscopically and parasitaemia determined. In all cases untreated cells were used as control. Curves were fitted by eyes.

4. Discussion

The normal shape of erythrocytes is an equilibrium state between two opposing shape changes(echinocytic and stomatocytic) maintained by many factors of which organization of the membrane skeleton, ATP levels, asymmetric lipid distributions, cholesterol/phospholipids ratio, pH gradient and the presence or absence of surface active agents are prominent[19, 24, 25]. It was

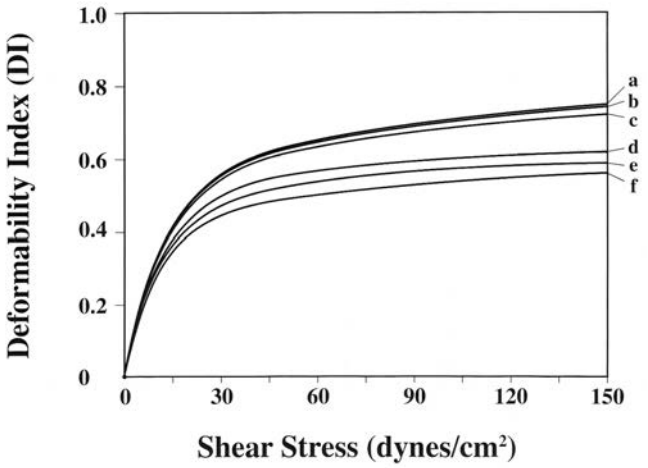


Fig. 3 Effects of echinocytogenic and stomatocytogenic agents on membrane deformability; DI of the control [untreated cells (line a)] and treated cells (b-f); (b) NaF, (c) PLA_2, (d) PLD, (e) SMase and (f) CPZ. Erythrocytes treated with stomatocytogenic agents showed decrease in DI_{max} ($\leq$ 0.6) whereas those treated with echinocytogenic agents also showed normal DI_{max} (>0.6).

observed in this study that both NaF and PLA_2 induced echinocytic change in erythrocytes whereas PLD, SMase and CPZ caused stomatocytic transformations. In both situations, Pf invasion was prevented due to loss of effective surface area required for membrane internalization. Using these agents (echinocytogenic and stomatocytogenic) to which erythrocytes respond by changing their shapes, our study has demonstrated that echinocytes and stomatocytes prevented Pf invasion supporting the observation that discoid shape of erythrocytes is essential for efficient Pf invasion.

The echinocytic transformation of hitherto discoid erythrocytes (Fig. 1 A) exhibited by NaF (Fig. 1 B) and PLA_2 (Fig. 1 C) treatments prevented Pf invasion (Fig. 2 A-B). The molecular mechanisms underlying this common echinocytic change vary. In the case of NaF, glycolytic enzymes were inactivated which led to the depletion of ATP with subsequent inactivation of flippase that translocates aminophospholipids such as PS from the outer to inner leaflet. The disruption of lipid asymmetry of the membrane in this way inevitably affected the inner leaflet interaction with membrane skeletal network[19]. In this case, random protrusions of the outer membrane occurred, making the cell echinocytic. Similarly, the echinocytic transformation induced by PLA_2 (Fig 1C) could be attributed to the generation of lysophospholipid and free fatty acids by hydrolysis of a PC[26] in the outer leaflet of the erythrocyte membrane. The accumulation of lysoPA and free fatty acids at the outer leaflet as suggested[27], expand the outer leaflet causing echinocytic transformation. It is also known that the abundance of cholesterol in the membrane appears to be maintained homeostatically near molar equivalence[28] and correct cholesterol/phospholipids ratio in the lipid bilayer helps to maintain the discoid shape of erythrocytes[25]. An irreversible PLA_2 degradation of phospholipids in the outer membrane probably increased the cholesterol/phospholipids ratio thereby contouring in the outward direction the membrane consistent with the finding[25]. The relatively uniform projections of the membrane, a characteristic of echinocytic transformation induced by NAF and PLA_2 are in opposite direction to membrane internalization. The effective surface areas that can be used for internalizations were reduced as result of the membrane projections, though extensibility and DI_{max} (Fig. 3 a-b) did not decrease. The reduced effective surface of the treated cells could account for the prevention of Pf invasion (Fig. 2 A-B) consistent with our hypothesis that large effective surface area is required for efficient Pf invasion into erythrocytes.

PLD, SMase and CPZ induced stomatocytic transformations when examined microscopically. It could be explained that PLD generates PA by hydrolysis of PC[29] and thus causing stomatocytic change (Fig. 1 D) of the erythrocytes. The observed stomatocytic transformation (Fig. 1 E) induced by SMase was consistent with the finding[30]. Zha[31] reported that treatment of cells with SMase rapidly induced the formation of numerous vesicles that pinch off from the plasma membrane generating endocytotic compartments. It is also known that sphingomyelin mainly distributed in the outer leaflet of the bilayer[32] is hydrolyzed thereby reducing its crowding in the outer leaflet, and the ceramide produced, just like the PA derived from PLD hydrolytic reactions could flip-cross the bilayer because of their hydrophobicity[30]. The movement of each metabolite (ceramide and PA) towards the inner leaflet could promote inward curvature of the membrane[14] making the red cell stomatocytic. SMase is also known to hydrolyse sphingomyelin from the membrane and this may destabilize the membrane lipid order, and subsequent transport of cholesterol into the inner leaflet could promote endocytotic change[31]. Movement of cholesterol

into the inner leaflet is likely to destabilize the cholesterol/phospholipids ratio in the outer leaflet, hence inward curvature[14, 25]. The endocytotic change in the erythrocyte induced by the SMase could also be explained in terms of the depletion of rafts (membrane microdomains) composed of cholesterol and sphingomyelin[33]. Stomatocytic transformation of human erythrocytes induced by CPZ (Fig. 1 F) was consistent with the finding of Sheetz and Singer[34]. CPZ (cationic amphipath) rapidly flip-cross the membrane in the neutral form and become charged by binding to PS (anionic phospholipids) and this leads to the expansion of the inner leaflet of the membrane inducing stomatocytic transformation[34]. The effective surface areas of these treated cells were reduced due to endocytotic change with decreased extensibility and DI_{max} (Fig. 3 d-f). This could account for the prevention of Pf invasion (Fig. 2 C-E) consistent with our hypothesis that large effective surface area is required for efficient Pf invasion into erythrocytes.

The normal erythrocyte by virtue of its biconcave discoid shape provides large effective surface area to enable the merozoite to randomly attach itself and rapidly brings the apical end in apposition to the erythrocyte surface. The merozoite entry into the red cell is associated with membrane internalization (invagination)[3, 4]. However, modified membranes in the cases echinocytes and stomatocytes, impaired the entry of the merozoite as result of the decrease in the effective surface area and/or the reduced extensibility of the membranes. In this case, internalization of the membrane was impaired impeding merozoite entry into the cell, hence prevention of Pf invasion in a concentration dependent manner (Fig. 2 A-E).

In conclusion, our study has demonstrated that echinocytic and stomatocytic erythrocyte shape changes prevent Pf invasion supporting the observation that biconcave discoid shape of erythrocytes with the large effective surface area is essential for Pf invasion and survival. This gives credence to the evolution of abnormal erythrocyte shapes which tend to limit Pf invasion, enlargement, multiplication and release of merozoites to complete erythrocytic life cycle[2]. Though, the use of enzymes to irreversibly manipulate the human erythrocytes is not ideal, the chemical agent (CPZ) with reversible behaviour on erythrocytes could be explored in sub-lethal levels for therapeutic properties in combination with other known anti-malarial drugs.

Abbreviations

Pf, *Plasmodium falciparum*; PLA_2, phospholipase A_2; PLD, phospholipase D; SMase, sphingomyelinase; NaF, sodium flouride; CPZ, chlorpromazine; PA, phosphatidic acid; lysoPA, lysophosphatidic acid; PC, phosphotidylcholine

Acknowledgement

We thank Dr Mohandas Narla at the New York Blood Center for useful suggestions. Ms Ito M. in our laboratory is highly appreciated for the technical assistance. The support given by Mark Ofosuhene in our laboratory is also acknowledged. This work was funded by Japan International Cooperation Agency (JICA) and supported by Grant-in-Aid for Scientific Research from the Ministry of Education Culture, Sport, Science and Technology of Japan (No. 16017296).

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(Received 28 December 2006 ;
Accepted 10 January 2007)