Transbilayer Phospholipid Asymmetry in *Plasmodium knowlesi*–Infected Host Cell Membrane

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Abstract. *The membranes from normal and* Plasmodium knowlesi–*infected rhesus monkey erythrocytes (90 to 95 percent infected with early ring stage) were analyzed for transbilayer distribution of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS), by means of chemical and enzymatic probes. The external monolayer of the normal red cell membrane contained at least 68 to 72 percent of the total phosphatidylcholine and 15 to 20 percent of the total phosphatidylethanolamine. In the infected cell, the transmembrane phosphatidylcholine distribution appeared to be reversed, with only 20 to 30 percent of it being externally localized, whereas roughly equal amounts of phosphatidylethanolamine were present in the outer and inner surfaces. However, total phosphatidylserine in both the infected and normal red cells was exclusively internal. Unlike that in the normal intact cell, external phosphatidylethanolamine in the parasitized cell was readily accessible to phospholipase* A_2. *These results indicate that significant changes in molecular architecture of the host cell membrane are the result of parasitization.*

Knowledge of molecular changes induced by the malaria parasite in the structural framework of the host cell membrane is essential to an understanding of host-parasite interactions. Various biochemical, biophysical, immunochemical (*1–4*), and electron microscopic studies (*5*) have revealed that the malarial parasite produces distinct morphological changes in the host erythrocyte membrane at the time of entry and during development.

Recently, Schmidt-Ullrich and Wallach (*3*) demonstrated the presence of *Plasmodium knowlesi*–specific antigens in the membrane of parasitized red cells of the rhesus monkey. However, few attempts have been made to determine the molecular changes occurring in the structure of host erythrocyte membrane integral proteins, glycolipids, and phospholipids. Alterations in the lipids of the red cell membranes in *Plasmodium lophurae* infections have been suggested by Holz (*6*). We have studied transbilayer phospholipid asymmetry in plasma membranes of rhesus monkey red cells that were 90 to 95 percent infected with the early ring stage of *P. knowlesi*. We report here a dramatic change in lipid organization in the membranes of infected monkey erythrocytes.

Synchronous infections of *P. knowlesi* (W 1 strain) were maintained by serial passage of infected blood in rhesus monkeys, caged in a room illuminated with fluorescent lights from 7:00 a.m. to 7:00 p.m. The monkeys were bled when parasites were at the early ring stage and when parasitemia was 90 to 95 percent, as determined by Giemsa staining. Blood was drawn into heparinized glass tubes and washed thrice with phosphate-buffered saline (PBS) (*p*H 7.2). White blood cells and platelets from normal blood, and white blood cells, platelets, and schizonts from the infected blood were removed by means of a Ficoll-Hypaque gradient, as described by Wallach and Conley (*2*). The purified infected erythrocytes, when checked by Giemsa staining, were 90 to 95 percent infected with early rings contaminated with 2 to 3 percent trophozoites.

Ghosts from normal or parasitized erythrocytes were prepared by lysing the cells with saponin (*7*). Normal red cell ghosts were isolated by centrifuging the saponized hemolyzate at $2 \times 10^4 g$. The hemolyzate from parasitized cells was centrifuged at $370g$ and the pellet was discarded. The supernatant was further centrifuged at $10^4 g$ to remove additional parasites. Finally, the membranes of the parasitized cells were isolated by centrifuging the supernatant derived from the later step at $4 \times 10^4 g$. The membranes prepared in this way were completely free of parasites as determined by light microscopy.

Extraction of lipids from the membranes was carried out according to the procedure of Folch *et al.* (*8*). Separation of various phospholipids was done on silica gel 60F-254 (with 0.25-mm, 20 by 20 cm, Merck glass plates) as described by Pollet *et al.* (9). Spots for different phospholipids were identified after staining the plate with iodine vapor followed by ninhydrin spray. These were removed and eluted with a mixture of methanol and chloroform (1:1 by volume) for several times including two overnight extractions. Total phosphorus present in each spot was determined as described in (10). The recoveries of various phospholipids from silica gel were 90 to 95 percent.

Phospholipase A_2, from *Naja naja*, and 2,4,6-trinitrobenzenesulfonic acid (TNBS), an amino group labeling reagent, were used as external probes to explore the distribution of phosphatidylcholine (PC) and aminophosphatides in plasma membranes of normal and parasitized rhesus monkey red cells. Extensive use of these probes has been made in studies of lipid asymmetry in other membrane systems (11, 12).

Incubations of intact cells and their unsealed membrane ghosts with phospholipase A_2 were carried out with varying concentrations of enzyme and for different time periods. Thc enzyme concentration that resulted in maximum hydrolysis of phospholipids in the intact cell, without any significant degree of hemolysis, was used in all experiments. The results are given in Table 1. The percentage of phospholipid degradation was calculated after estimating the amounts of lysophospholipids and intact phospholipids. In the normal cell, 68 to 72 percent of the total PC was hydrolyzed, whereas no degradation of phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) was observed. In intact infected erythrocytes, only PC (21 to 27 percent) and PE (44 to 48 percent) were hydrolyzed. An increase in the incubation time (90 minutes) did not lead to further increases in the extent of PC hydrolysis in the normal cell or PC and PE degradation in the parasitized cell. There was complete degradation of PC, PE, and PS (95 to 100 percent) in unsealed membrane ghosts of normal and parasitized cells.

Normal intact cells were treated with TNBS according to established procedures (13–15). Fifteen to 20 percent of the PE was modified (Table 2). However, infected cells were strongly agglutinated if the reaction was carried out by any of the above methods. The resultant sheath-like structure of cells resisted lysis by most of the known cell lysis procedures. The modified reaction medium (Table 2) eliminated this problem. Treatment of the parasitized cells with TNBS in the modified medium resulted in 45 to 50 percent modification of PE. Since there was no difference in the extent of TNBS labeling in experiments carried out for 6 hours and 12 hours, it was inferred that the reagent did not cross the membrane under these experimental conditions. Reaction of unsealed membrane ghosts with TNBS gave complete labeling of PE and PS.

These results indicate that at least 68 to 72 percent of PC, 15 to 20 percent of PE, and zero percent of PS have an external localization in the normal uninfected rhesus monkey erythrocyte membrane; this is similar to the arrangement of phospholipids in the human red cell (16). On the basis of the known external localization of sphingomyelin in the human red cell, it might be supposed that a similar arrangement of this phospholipid is present in the monkey erythrocyte. However, contrary to the normal red cell, at least 21 to 27 percent of PC, 45 to 50 percent of PE, and zero percent of PS were distributed in the outer monolayer of the parasitized cell membrane, constituting roughly 20 percent of the total phospholipids (17).

These findings demonstrate that dramatic changes in molecular organization of the phospholipid bilayer of the red cell membrane are induced by malaria para-

Table 1. Nonlytic degradation of phospholipids in normal and *P. knowlesi*–infected rhesus monkey erythrocytes and their unsealed ghosts by phospholipase A_2. Phospholipase A_2 from *Naja naja* snake venom (Haeffkin Institute, Bombay) was purified essentially according to the method of Blecher (20). The resulting concentration of the protein was approximately 1 mg/ml. Treatments of the normal and the infected erythrocytes as well as of their unsealed ghosts with phospholipase A_2 were carried out according to the method of Verkleij *et al.* (16). The amount of enzyme used was 70 μl of the stock solution per 0.25 ml of packed cells and ghosts. Incubations were done for 60 minutes at 37°C. Enzyme activity was inhibited by addition of *o*-phenanthroline and EDTA (16). The cells and ghosts were washed twice with isotonic saline prior to the lipid extraction. The percentage of degradation of phospholipids was determined by estimation of total phosphorus content in various phospholipid spots. Values are expressed as mean of a number of determinations (N) ± standard deviation or as a range. Percentage hemolysis was determined as described by Roelofsen *et al.* (21). Three to 5 percent of the normal cells and 4 to 7 percent of the infected cells were lysed under these conditions; N.D., not determined.

Substrate	Percentage of degradation of phospholipids					
	Total phospholipids	PC	PE	SM	PS	PI
Normal intact cell (N = 6)	26.84 ± 1.79	68 to 72	0	0	0	0
Infected intact cell (N = 6)	19.48 ± 1.23	21 to 27	44 to 48	0	0	0
Normal cell ghost (N = 3)	82.38 ± 1.76	100	100	0	100	N.D.
Infected cell ghost (N = 3)	71.15 ± 1.47	100	100	0	100	N.D.

Table 2. Results of labeling with TNBS the aminophosphatides in normal and *P. knowlesi*–infected intact rhesus monkey erythrocytes and their unsealed ghosts. The labeling of normal erythrocyte was carried out according to Gordesky and Marinetti (13), Bonsall and Hunt (14), and Gordesky *et al.* (15). Use of the last two procedures resulted in similar amounts of labeling of PE, whereas the first one gave 2 to 3 percent less labeling in the intact cell. Values for total phospholipid labeling were obtained by following the last procedure. The infected cells were strongly agglutinated if the labeling was carried out by either of the above procedures. However, inclusion of 1 m*M* EDTA and 2.0 U/ml of preservative-free heparin into the medium used by Gordesky *et al.* (15) was found to be satisfactory. No detectable change in the amount of labeling of aminophosphatides in normal intact cells was observed by using this modified medium. The reactions were carried out at 23° to 25°C for 6 to 12 hours. No differences in percentage of labeling in these experiments were observed. No detectable cell lysis occurred in all these experiments. The percentage of TNBS labeling was determined by the total phosphorus determinations as well as by measuring absorbance of yellow color at 340 nm. Values are expressed as mean of a number of determinations (N) ± standard deviation or as a range.

Cell or ghost	Percentage of TNBS labeling of phospholipids		
	Total phospholipids	PE	PS
Normal intact cell (N = 6)	5.95 ± 0.65	15 to 20	0
Infected intact cell (N = 4)	10.07 ± 0.50	45 to 50	0
Normal cell ghost (N = 3)	44.78 ± 1.32	100	100
Infected cell ghost (N = 3)	33.72 ± 1.40	100	100

site. Since the lipid asymmetry seems to be a result of differential binding of phospholipids by proteins and other ligands on the two sides of the membrane (*18*), alterations in molecular organization of the lipid bilayer could induce changes in distribution of other membrane components. The suggested aggregation of the integral proteins of the host cell membrane during *P. knowlesi* infection (*5*) could therefore be a result of altered phospholipid asymmetry. The complete hydrolysis of the external PE in parasitized cells by phospholipase A_2 also suggests that the protein distribution in the host cell membrane is altered.

It is important to consider how the observed changes in composition and inside-outside distribution of phospholipids in the infected host cell membrane come about. We suggest that the parasite may secrete phospholipid-rich vesicles on the host cell surface at the time of its entry into the cell. These vesicles may then fuse with the host erythrocyte membrane causing changes in composition and assembly of phospholipids. The presence of lipid vesicle-like structures on the host cell surface has already been demonstrated at the time of the merozoite's entry into the cell (*5, 19*).

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References and Notes

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