An intracellular simian malarial parasite (*Plasmodium knowlesi*) induces stage-dependent alterations in membrane phospholipid organization of its host erythrocyte

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The membrane phospholipid organization in monkey erythrocytes harbouring different developmental stages of the simian malarial parasite *Plasmodium knowlesi* was studied using phospholipase A₂ from two different sources and Merocyanine 540 as the external-membrane probes. Experiments were done to confirm that the phospholipases did not penetrate into the infected cells or hydrolyse phospholipids during membrane isolation. The parasite-free erythrocyte membrane was isolated by differential centrifugation or by using the cationic beads Affi-Gel 731. The purity of the membranes was established by optical and electron microscopy, and by assaying the parasite-specific enzyme glutamate dehydrogenase. About 10% of the phosphatidylethanolamine and none of phosphatidylserine were hydrolysed by the phospholipases in intact normal monkey erythrocytes. However, accessibility of these aminophospholipids to the enzymes was significantly enhanced in the infected cells under identical conditions. The degree of this enhancement depended on the developmental stage of the intracellular parasite, but not on the parasitaemia levels in the infected monkeys, and increased with the parasite growth inside the cells. Analogously, Merocyanine 540 was found to label the trophozoite- or schizont-infected erythrocytes, but not the ring-infected or normal cells. These results demonstrate that the intracellular malarial parasite produces stage-dependent alterations in the membrane phospholipid organization of its host erythrocyte.

INTRODUCTION

Malarial parasite grows and multiplies within the erythrocytes of an infected host during blood stage of the infection. To derive its nutrients from plasma, the intracellular parasite modifies the erythrocyte membrane permeability properties (Kutner et al., 1983; Ginsburg et al., 1985), presumably by altering the structure and organization of the membrane components. An understanding of these alterations is therefore essential to gain a deeper insight into host–parasite interactions at the molecular level.

Earlier studies have shown that the intracellular parasite induces marked structural changes in the host-cell-membrane phospholipids and proteins (Sherman, 1985). It modifies the fatty acyl composition of the membrane phospholipids (Holz, 1977) and also the structure of carbohydrates bound to the membrane integral proteins (Shakespeare et al., 1979; Vincent & Wilson, 1980; Trigg et al., 1977). Furthermore, there is evidence to suggest that the membrane-associated cytосkeletal proteins are degraded at least at the schizont stage of the parasite maturation (Weidekamm et al., 1973; Wallach & Conley, 1977; Yuthavong et al., 1979). Apart from these alterations, the parasite is known to influence the membrane fluidity (Howard & Sawyer, 1980; Allred et al., 1983; Sherman & Greenan, 1984) as well as the membrane cholesterol levels (Holz, 1977). These changes in the structure and compositions of the membrane components should influence the lipid–lipid and lipid–protein interactions within the erythrocyte membrane bilayer, which in turn would affect the membrane phospholipid organization (Demel & de Kruyff, 1976; Haest, 1982). However, only a few attempts (Gupta & Mishra, 1981; Taraschi et al., 1986) have been made to analyse these effects at the molecular level. Here we describe the results of our studies on the phospholipid organization in the membranes of rhesus-monokey erythrocytes that harboured different developmental stages of the simian malarial parasite *Plasmodium knowlesi*. Phospholipase A₂ from two different sources and Mc 540 were employed as the external-membrane probes. Experiments were done to confirm that the enzymes did not penetrate into the cells or hydrolysed the infected erythrocyte membrane phospholipids during the host-cell-membrane isolation. Results of these studies indicate that the intracellular malarial parasite induces marked alterations in host-cell-membrane aminophospholipid (PE and PS) organization, depending on the stage of its development inside the cells.

EXPERIMENTAL

Materials

Healthy rhesus monkeys of either sex, weighing 4–6 kg, were procured from the Primate House of our Institute. Bee-venom and porcine pancreatic phospholipases A₂ were purchased from Sigma. Mc 540 was bought from

Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; Mc 540, Merocyanine 540; TNBS, trinitrobenzenesulphonic acid; PBS, phosphate-buffered saline (5 mm-phosphate/150 mm-NaCl, pH 7.4).

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Boehringer-Mannheim. Affi-Gel 731 was from Bio-Rad Laboratories. Ficoll 400 and Conray 420 were procured from Pharmacia Fine Chemicals and May and Baker respectively. Pre-coated silica-gel 60F-254 plates (20 cm x 20 cm; 0.2 mm thickness) were obtained from E. Merck. Na\(^{14}\)I and iodo\(^{14}\)C)methane were obtained from Bhabha Atomic Research Centre, Trombay, India.

Egg PC and egg [methyl-\(^{14}\)C]PC (sp. radioactivity 30 \(\mu\)Ci/\(\mu\)mol) were prepared as described previously (Gupta & Bali, 1981). Pancreatic phospholipase \(A_2\) was radiolabelled with \(^{125}\)I essentially by the method of Litman et al. (1980). Egg PC liposomes containing traces of egg \([^{4}\)C]PC were prepared by sonication and fractionated by ultracentrifugation (Gupta & Bali, 1981).

**P. knowlesi infection in monkeys**

Synchronous infections of *P. knowlesi* were maintained by serial passage of infected blood in healthy rhesus monkeys, caged in a room illuminated with fluorescent light from 07:00 h to 19:00 h. The monkeys were bled at different levels of parasitaemia. Parasitaemias were determined by counting 10\(^4\) erythrocytes in a thin blood smear stained with Giemsa stain and expressed as number of parasitized cells/100 erythrocytes.

**Isolation of infected erythrocytes**

Blood from infected monkeys was collected in heparinized glass tubes. After removing the plasma, the cells were diluted with equal volume of PBS, and loaded on a Ficoll–Conray gradient [density (\(\rho\)) 1.076 g/cm\(^3\)], which was prepared as described previously (Singhal et al., 1986). The ratio of the gradient to the cell suspension was 2:1 (v/v). It was centrifuged at 50 g for 10 min (20 °C). The schizont-infected cells floated on the top, were carefully removed and washed three times with PBS. Leucocytes from these cells were removed by using a second gradient of Ficoll–Conray (\(\rho\) 1.08 g/cm\(^3\)) at 350 g. The schizont-infected cells thus obtained were contaminated with 5–7\% uninfected erythrocytes, less than 1\% ring-infected cells and less than 0.2\% leucocytes. The trophozoite-infected cells were enriched by twice using the Ficoll–Conray gradient (\(\rho\) 1.076 g/cm\(^3\)). These cells were contaminated with 10–15\% uninfected cells, 5–10\% ring-infected cells and less than 0.1\% leucocytes. Finally, the ring-infected cells were enriched to 70–75\% by using the method described above for the trophozoite-infected erythrocytes.

The infected erythrocytes thus isolated were largely intact, as determined by optical microscopy (Fig. 1). In some cases, however, less than 5\% schizont-infected cells and less than 2\% trophozoite (or ring)-infected erythrocytes were lysed during the experimental handling.

**Treatment of erythrocytes with phospholipase \(A_2\)**

A 0.25 ml portion of packed cells was suspended in 5.0 ml of 10 mM-glycylglycine buffer containing 100 mM-KCl/50 mM-NaCl/0.25 mM-MgCl\(_2\)/0.25 mM-CaCl\(_2\)/44 mM-sucrose, pH 7.4. To it was added 5 i.u. of bee-venom phospholipase \(A_2\), and the mixture incubated for 15 min at 37 °C. The enzyme reaction was stopped by washing the cells three times with 0.9\% NaCl containing 5 mM-EDTA. The extent of haemolysis was determined at the end of each incubation before the EDTA wash by comparing the haemoglobin content in the supernatant of each sample with that of a 100\%-haemolysed control. Haemolysis was less than 5\%.

Treatments of erythrocytes with porcine pancreatic phospholipase \(A_2\) (10 i.u./0.25 ml of packed cells) were carried out for 15–45 min at 37 °C in the above incubation medium, except that the CaCl\(_2\) concentration was increased from 0.25 mM to 10 mM. The extent of haemolysis during the enzyme reactions was less than 3\%.

Unsealed erythrocyte ghosts, prepared as described below, were treated with both bee-venom and pancreatic phospholipase \(A_2\) essentially under the conditions described above for the intact cells.

**Isolation of parasite-free erythrocyte membranes**

The enzyme-treated or untreated infected cells were lysed with 20 mM-phosphate (Wiser et al., 1983) containing 0.1 mM-EDTA at 4 °C and the lysate was immediately centrifuged at 750 g for 2 min (4 °C). The supernatant was carefully aspirated off, leaving behind a pellet consisting of intact parasites and a few unlysed cells. Further removal of the parasites from the supernatant was accomplished by centrifugation at 2250 g (3 min, 4 °C). Finally, the membranes were isolated by centrifuging the second supernatant at 20000 g for 45 min (4 °C). Alternatively, the parasite-free host erythrocyte membranes were isolated as described by Gruenberg & Sherman (1983) using Affi-Gel 731. The membranes thus obtained were virtually free from intact parasites, as judged by optical and transmission electron microscopy, and by assaying the parasite-specific enzyme glutamate dehydrogenase (Vander Jagt et al., 1982).

**Parasite lysis determination**

The lytic effect of 20 mM-phosphate on the parasites was determined as follows. The infected erythrocytes were lysed as described above, and the lysate was immediately centrifuged at 2250 g for 3 min. The pellet
Plasmodium knowlesi-induced changes in host erythrocyte

Table 1. Erythrocyte membrane phospholipid composition

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>Composition (%)</th>
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<tbody>
<tr>
<td></td>
<td>PC</td>
</tr>
<tr>
<td>Normal</td>
<td>38.8±1.1</td>
</tr>
<tr>
<td>Ring-infected</td>
<td>39.5±1.6</td>
</tr>
<tr>
<td>Trophozoite-infected</td>
<td>39.0±1.6</td>
</tr>
<tr>
<td>Schizont-infected</td>
<td>39.4±2.5</td>
</tr>
</tbody>
</table>

was washed three times with PBS and then resuspended in the same buffer. A measured portion of the suspension was analysed for glutamate dehydrogenase activity (Vander Jagt et al., 1982). The remaining suspension was centrifuged at 15000 g (15 min, 4°C), and the pellet treated with 20 mM-phosphate. The parasites were harvested by centrifugation and assayed for glutamate dehydrogenase activity (Vander Jagt et al., 1982). The specific activity of the enzyme remained virtually unaffected by the 20 mM-phosphate treatment, indicating that this treatment does not lyse the parasite significantly. However, treating the isolated parasites with 0.2% saponin (Rock et al., 1971) resulted in a 60–80% loss of the enzyme activity.

Lipid extraction

Extraction of lipids from the membranes was carried out by the method of Rose & Oklander (1965). In the case of membranes that were isolated by the Affi-Gel method, the bead-bound membranes were directly used for lipid extraction. That the extraction efficiency of the membrane phospholipids is not affected by binding of the membranes to the Affi-Gel beads was confirmed by determining their relative amounts in the bead-bound membranes and then comparing these amounts with those observed in the lipid extracts of membranes isolated by differential centrifugation. Lipids from the enzyme-treated normal uninfected monkey erythrocytes were isolated by extracting whole cells (Kumar & Gupta, 1983) or membranes prepared by the Affi-Gel method or by centrifugation.

Determination of phospholipid degradation

The lipid extracts prepared as described above were evaporated to dryness under N₂. The residue was dissolved in a small volume (100–200 µl) of chloroform/methanol (2:1, v/v) mixture. Individual phospholipids were separated by two-dimensional t.l.c. as described previously (Pollet et al., 1978). Spots from different phospholipids were identified after staining the plate with I₂ vapour followed by ninhydrin spray. These were removed and eluted several times with chloroform/methanol (1:1, v/v). Total phosphorus was determined as described by Ames & Dubin (1960). The recoveries of various phospholipids from silica gel were more than 95%.

Mc 540 labelling

Labelling of erythrocytes with Mc 540 was carried out by the method of Schlegel et al. (1980). The cells were suspended to a concentration of about 4 x 10⁸ cells/ml of Tris-buffered saline (10 mM-Tris/140 mM-NaCl/10 mM-KCl/3 mM-MgCl₂, pH 7.4) containing 5% pooled monkey serum and Mc 540 (14 µM). The mixture was incubated at 37°C for 10 min. Fluorescence microscopy was performed with Polyvar fluorescence optical microscope.

RESULTS

The host-cell membrane from the infected erythrocyte was isolated by differential centrifugation or by using Affi-Gel 731, and the relative amounts of the major phospholipids (namely PC, SM, PE and PS) determined. Results given in Table 1 indicate that the membrane phospholipid composition in erythrocytes is not significantly influenced by their parasitization with P. knowlesi. To examine whether the transbilayer phospholipid distribution is affected in the infected erythrocytes, we analysed the accessibility of the various glycerophospholipids to both bee-venom and pancreatic phospholipases A₂ in intact normal as well as in infected cells. Table 2 shows that bee-venom phospholipase A₂ did not hydrolyse PS in the normal uninfected monkey erythrocytes, but the same enzyme under identical conditions readily degraded this aminophospholipid in the infected erythrocytes. The extent of this phospholipid degradation varied with the maturation stage of the intracellular parasite. Maximum PS degradation occurred in the schizont-infected cell (~ 43%), whereas it was minimum in the ring-infected erythrocytes (~ 13%). Also, the amounts of hydrolysed PE varied in that order. About 28, 34 and 44% of this lipid were degraded by the enzyme in the ring-, trophozoite- and schizont-infected cells respectively.

PS hydrolysis in the infected erythrocytes was further confirmed by using pancreatic phospholipase A₂ as the enzymic probe. The data shown in Table 3 indicate that this enzyme did not attack the normal cells, but, like the bee-venom phospholipase A₂, it readily degraded PS in the infected erythrocytes. Also, this degradation increased with the parasite's development from the ring to the schizont stage. These results clearly indicate that accessibility of the aminophospholipids to phospholipases A₂ in the intact erythrocyte is enhanced upon their parasitization with P. knowlesi.

Enhancement of aminophospholipid hydrolysis is not due to penetration of the enzyme into the infected cells, as suggested by our finding that these enzymes under identical conditions hydrolysed significantly greater amounts of the various glycerophospholipids in the unsealed infected erythrocyte ghosts (Tables 2 and 3). This was further confirmed by determining the residual amounts of cell-associated pancreatic phospholipase A₂.
after the enzyme treatments. Both the normal and schizont-infected erythrocytes were incubated with $^{125}$I-labelled pancreatic phospholipase $A_2$ essentially under the conditions given in the Experimental section. The enzyme-treated washed cells were counted for radioactivity and then lysed with distilled water. The lysates were centrifuged at 20000 × $g$ (60 min), and the radioactivity was determined in both the pellet and supernatant. About 0.6% and 0.8% of the total $^{125}$I were found to be associated with the normal and infected cells respectively. Of these amounts, ~90% of the $^{125}$I was present in the pellet and ~10% in the supernatant of both the types of cells. These findings clearly indicate that the phospholipases do not penetrate into the schizont-infected erythrocytes, and that only the external glycerophospholipids are hydrolysed in both the normal and infected cells under our experimental conditions.

To examine whether the residual enzyme activity associated with the washed phospholipase $A_2$-treated cells would cleave phospholipids during membrane isolation, we incubated egg-$^{[14]}$C]PC-labelled liposomes with the isolated infected erythrocyte membranes at 37 °C for 60 min. No degradation of egg $^{[14]}$C]PC was detected under these conditions. Moreover, the amounts of degraded phospholipids in the enzyme-treated normal cells remained unaltered when determined by whole-cell lipid extraction or by extracting lipids from the isolated membranes. This completely rules out the possibility of phospholipid hydrolysis during membrane isolation.

These results indicate that both bee-venom and pancreatic phospholipases $A_2$ hydrolyse only the external glycerophospholipids in the intact cells. Since these enzymes degraded significantly greater amounts of the aminophospholipids in the intact infected erythrocytes as compared with the normal uninfected cells, it would seem that the enhanced phospholipid hydrolysis is probably due to malarial-parasite-induced structural perturbations in the host erythrocyte membrane. The degree of perturbation appears to depend on the developmental stage of the intracellular parasite rather than on the parasite load in the infected monkeys, as accessibility of PE and PS to the enzymes was found to increase with the parasite maturation inside the cells but not with the parasitaemia levels in the animals (Tables 2 and 3). This is quite consistent with a recent study (Taraschi et al., 1986) showing that the intracellular human malarial parasite (P. falciparum) produces stage-dependent structural alterations in the host-cell-membrane phospholipids.

To establish further that the membrane structure is impaired in the infected cells, we analysed the membrane fluidity by means of a 'fluid-sensing' fluorescent dye, Mc 540, as the external-membrane probe (Williamson et al., 1982). Fig. 2 shows that this dye readily labelled both the trophozoite- and schizont-infected erythrocytes, but not the ring-infected or the normal uninfected cells, which is quite in accordance with a previous study (Sherman & Greenan, 1984). The virtual failure of Mc 540 to label the
ring-infected cells may largely be attributed to the qualitative nature of the assay, which makes it seemingly difficult to detect small increases in the outer-surface fluidity (Williamson et al., 1985).

In our previous study (Gupta & Mishra, 1981) we could not detect PS in the outer surface of the ring-infected cells by means of snake (Naja naja)-venom phospholipase A₂ and TNBS. This variation from the present results may primarily be attributed to the differences in the methods that we employed in the two studies for isolating the host erythrocyte membrane and also for probing the membrane lipid organization. We have previously used 0.2% saponin for lysing the infected cells during membrane isolation, which in this and other (Ancelin & Vial, 1986) studies has been found to lyse the parasites as well. Also, the enzymic probes employed in the two studies have different substrate specificities. Whereas Naja naja phospholipase A₂ is known to cleave PE faster than PC and PS, phospholipase A₂ from porcine pancreas hydrolyses PS faster than PC and PE (Roelofsen, 1982). Besides, the use of TNBS for probing the membrane aminophospholipid organization has now been considered unsatisfactory (Haest et al., 1981).

Previous studies have shown that, besides the parasitized cells, the non-parasitized erythrocytes of P. knowlesi-infected monkeys also contain the procoagulant phospholipid PS in the outer surface of their membrane bilayer (Gupta et al., 1982). It may, therefore, be argued that the observed membrane abnormalities in the ring-infected cells do not arise from parasitization but could have resulted from contamination of these cells with the non-parasitized erythrocytes. To investigate this possibility, we analysed the membrane phospholipid organization in the non-parasitized erythrocytes of monkeys infected with the schizont stage of P. knowlesi, essentially under the conditions as described in the present study for the parasitized erythrocytes. Only 5–14% of the PS and 18–25% of the PE were accessible to the phospholipases in the intact cells. This abnormality in the non-parasitized cells has, however, recently been shown not to originate from the presence of malarial parasite in the blood but primarily to arise from the malaria-induced secondary complications (Joshi et al., 1986). It may therefore be suggested that the observed abnormalities in the ring-infected cells are not solely due to the contamination with the non-parasitized erythrocytes.

**DISCUSSION**

Phospholipases have been extensively used as a tool to analyse the membrane phospholipid organization in the erythrocyte membrane (Roelofsen, 1982). Although the extent to which these enzymes cleave various phospholipids in the intact cell has usually been correlated with the extent of their localization in the external monolayer (Roelofsen, 1982), it has recently been suggested that, in some cases, the phospholipase treatment may induce transbilayer relocation of phospholipids in the modified erythrocytes (Op den Kamp et al., 1985; Franck et al., 1986). It is therefore difficult at the moment to ascertain whether the malarial parasitization of the erythrocytes leads to partial migration of the membrane aminophospholipids to the outer monolayer or if simply increases the 'flip–flop' rates of the various phospholipids, without altering the transbilayer lipid distribution. Since either of these cases would represent an organizational change in the erythrocyte membrane phospholipids, we conclude that the intracellular P. knowlesi produces stage-dependent alterations in the membrane phospholipid organization of the host cells.

Normal erythrocytes contain choline phospholipids mainly in the outer half of their membrane bilayer, whereas the aminophospholipids are localized almost exclusively in the inner monolayer (Op den Kamp, 1979). This typical asymmetric transbilayer phospholipid distribution in these cells appears to be maintained by the interactions between the inner-layer phospholipids and the cytoskeletal proteins (Haest, 1982; Sato & Ōnishi, 1983; Bonnet & Begard, 1984; Cohen et al., 1986). Also, an ATP-driven aminophospholipid transport from the outer to the inner monolayer has been implicated in maintaining the inner distribution of PE and PS in the erythrocyte membrane (Seigneuret & Devaux, 1984; Zachowski et al., 1985, 1986). This transport has, however, been shown to be inhibited by an increase in the erythrocyte cytoplasmic Ca²⁺ concentration (Zachowski et al., 1986). Since an intracellular malarial parasite seems to degrade the host erythrocyte cytoskeletal proteins (Weidekamm et al., 1973; Wallach & Conley, 1977; Yuthavong et al., 1979) and has also been demonstrated to induce a substantial increase in the infected cell Ca²⁺ levels (reviewed by Sherman, 1985), it may be suggested that the observed changes in the membrane phospholipid organization may have been caused by these alterations in the parasitized red cells.

The transbilayer phospholipid movement (flip–flop) in the native erythrocyte membrane is relatively a slow process (Van Meer & Op den Kamp, 1982). But this movement is considerably enhanced in the red cells that possess an altered cytoskeleton (Franck et al., 1983, 1985, Mohandas et al., 1982, 1985; Bergmann et al., 1984). As the malarial parasite appears to produce marked structural changes in the host-cell cytoskeleton during (McLaren et al., 1979; Aikawa et al., 1981) and after (Weidekamm et al., 1973; Wallach & Conley, 1977; Yuthavong et al., 1979) invasion, it may be considered that these cytoskeletal alterations could result in an
increase in the rate of phospholipid translocation across the erythrocyte membrane.

Finally, it is important to consider the reasons for which the intracellular malarial parasite produces the observed membrane lipid changes. These changes are perhaps required by the parasite to recondition the host erythrocyte membrane structure and function to its needs. On one hand, an altered organization of membrane lipids may be expected to modify the erythrocyte membrane permeability, whereas, on the other, it may lead to changes in the organization and consequently the function of the membrane proteins. It is therefore possible that the new permeability pathways observed (Kutner et al., 1983; Ginsburg et al., 1985) in the infected-cell membrane could have resulted from the membrane lipid changes in the parasitized cells. Also, these changes might have been required to provide an appropriate lipid environment to the new proteins that the parasite inserts into the host-cell membrane bilayer (Sherman, 1985).

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