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Phospholipid uptake by *Plasmodium knowlesi* infected erythrocytes

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The uptake of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) in *Plasmodium knowlesi* infected erythrocytes has been studied. Whereas uptake of phospholipids, in the absence of phospholipid transfer proteins, is negligible in control cells, the infected cells can incorporate considerable amounts of added phospholipids. The uptake is enhanced by the presence of lipid transfer proteins. Doubly labeled [^{3}H]oleate, [^{14}C]choline) PC does not undergo any appreciable remodelling following uptake, which strongly suggests that plasma PC is used as such for the biogenesis of the parasite membranes. Transport of extracellularly offered PS and PE towards the intraerythrocytic parasite and utilization of these lipids by the parasite are confirmed by the observation that these lipids are converted into respectively PE and PC. The extent and rate of these conversions depend on the way the phospholipids are introduced into the infected cells.

Malaria; Phospholipid transfer protein; Phospholipid turnover; (*Plasmodium knowlesi*)

1. INTRODUCTION

Following infection by the malarial parasite *Plasmodium knowlesi*, erythrocytes become enriched in phospholipid content. In the last stage of parasite development, infected cells contain 3–5 times more phospholipids than uninfected erythrocytes [1,2]. Infected erythrocytes contain phospholipid synthesizing enzymes [3], which are absent in uninfected erythrocytes [4]. The parasite is however unable to synthesize fatty acids, which are needed for the biosynthesis of lipids and it has been shown previously that free fatty acids can be incorporated directly [2,3]. Also phospholipid molecules can be transferred to the parasite as was demonstrated by experiments in vitro using a PC specific transfer protein [5]. The present experiments were designed to answer the question if these PC molecules can be transferred to the parasite in an intact form to be subsequently incorporated as such, or whether the parasite might acquire fatty acids by hydrolysis of the incorporated PC. To discriminate between the various possibilities, double-labeled PC containing [^{3}H]-oleate and [^{14}C]choline was introduced into the cells. After the incubation we determined the isotope ratios in the PC recovered from the infected cells as well as the transfer of radioactivity to other lipids. Similarly uptake and conversion of radiolabeled vesicle PS and PE was studied both in the presence and absence of transfer protein.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

[^{3}H]Oleic acid, phosphatidyl[U-^{14}C]serine and phosphatidyl[2-^{14}C]ethanolamine were obtained from Amersham International, England. Phosphatidyl[*methyl*-^{14}C]choline was synthesized according to Stoffel [6]. 1-Acyl-2-[^{3}H]oleoyl-*sn*-PC was synthesized by coupling of [^{3}H]oleic acid anhydride and lysoPC. Egg PC, egg PA and cholesterol were obtained from Sigma (St. Louis, MO, USA)

2.1.1. Preparation of [^{3}H]oleic acid anhydrides

Chloroform was dried on P_2O_5 and distilled twice. Tritium-labeled oleic acid was dissolved in 0.1 ml of dry chloroform and a solution of *N,N*-dicyclohexylcarbodiimide (DCCD) in chloroform was added in a molar ratio of oleic acid/DCCD = 2:1. The synthesis was followed by thin-layer chromatography

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on silica gel plates (Merck Fertigplatten) using diethyl ether/petroleum ether (1:4, v/v) as eluents. The R_f values of oleic acid and anhydride were 0.59 and 0.96, respectively. The anhydride was scraped off the plate and eluted from the silica gel with diethyl ether/petroleum ether (1:4, v/v).

2.1.2. Acylation of lysophosphatidylcholine

Egg lysoPC was dissolved in 0.1 ml dry chloroform and added to a mixture of the labeled oleic acid anhydride to be coupled with 4-(dimethylamino)pyridine (DMAP). This catalyst was used in a molar ratio of lysoPC/DMAP/oleic acid anhydride of 1:1.1:1.1. The reaction mixture was incubated for 24 h at room temperature under nitrogen. This was followed by thin-layer chromatography on silica gel plates, first with diethyl ether/petroleum ether (1:4, v/v) as eluents in order to remove oleic acid anhydride and after that with $CHCl_3$/CH_3OH/H_2O/NH_3 (90:54:5.5:5.5, v/v). The PC was eluted from the silica with $CHCl_3$/CH_3OH (1:4, v/v).

2.2. *Production and collection of* Plasmodium knowlesi *infected erythrocytes*

Rhesus monkeys (*Macaca mulatta*) were obtained from Shamrock Farm Henfield, Sussex, England and Cynomolgus monkeys (*Macaca fascicularis*) from Sanofi Research Center (Montpellier, France). They were fed ad libitum with artificial diet (no.107 from UAR, Epinay sur Orage, France). All animals had been splenectomized and were susceptible to infection after 6 weeks of convalescence.

Monkeys were infected with *Plasmodium knowlesi* (Washington strain, variant 1, Dr G. Mitchell, Guy's Hospital Medical School, London) by an intravenous inoculation with $2–6 \times 10^8$ infected cells, previously cryopreserved according to Rowe et al. [7] or from animal to animal. Levels of parasitemia (i.e. the percentage of infected erythrocytes) were determined at appropriate intervals by microscopic examination of a blood smear stained with Giemsa azure type B. On post-infection days 6–10, blood was removed by venipuncture and collected in citric acid/dextrose after the monkey had been anesthetized by an intramuscular injection of ketamine (Iffa-Credo, France). Control blood was obtained in a similar way. Infected monkeys were cured by intramuscular injection of 150 mg chloroquine on 3 consecutive days.

Cells were collected by centrifugation at 7000 × *g* and suspended in RPMI 1640 supplemented with 25 mM Hepes (pH 7.4). White cells were removed by passage through a cellulose powder column (Whatman CF11) [8]. Separation of infected and uninfected red blood cells was performed by the Percoll-sorbitol fractionation as described by Kutner et al. [9]. After enrichment, parasitemia was in the range of 95–100% and white blood cells were practically absent, as monitored by microscopic examination of smears.

2.3. *Isolation of transfer proteins*

A non-specific lipid transfer protein was isolated from bovine liver as described in [10]. Before use, it was dialyzed against an adequate volume of buffer containing 280 mM sucrose, 10 mM Tris, 1 mM EDTA, 10 mM NaCl, 20 mM glucose, 100 IU penicillin G/ml and 0.1 mg streptomycin/ml.

The phosphatidylcholine specific transfer protein was prepared from beef liver and purified according to Westerman et al. [11]. Before use, the required amount of protein was dialyzed against a buffer containing 150 mM NaCl, 25 mM glucose, 1 mM EDTA, 0.02% NaN_3 and 10 mM Tris-HCl at pH 7.4 to remove glycerol. It was concentrated over polyethylene glycol flakes to the required concentration.

2.4. *Lipid analysis*

Phospholipids were extracted from intact cells according to Folch et al. [12]. Lipids were separated by thin-layer chromatography as described by Broekhuyse [13]. Radioactivity was measured in 299 TM emulsifier scintillation solution from Packard, using a Packard-PRIAS-Tricarb scintillation counter. Phosphorus was determined according to Rouser et al. [14].

2.5. *Incubation with non-specific lipid transfer protein (nsLTP)*

Cells were washed twice in the incubation buffer which contained 280 mM sucrose, 10 mM NaCl, 20 mM glucose, 10 mM Tris and 1 mM EDTA at a pH of 7.4. Vesicles containing PC, PA, cholesterol and phosphatidyl[U-^{14}C]serine (0.4:0.01:0.4:0.78 molar ratio) were prepared as described by Kuypers et al. [15]. Similar vesicles were prepared containing phosphatidyl-[2-^{14}C]ethanolamine (0.5 mol%). Incubations were carried out with a 10–20-fold excess of erythrocyte membrane phospholipid over vesicle phospholipid. Exchange of lipids between vesicles and erythrocytes was stopped by washing three times in the buffer described above, thus removing vesicles and nsLTP. Incubation was carried out at 37°C at a hematocrit of 10–30.

2.6. *Incubation with the PC specific transfer protein (PC-TP)*

Red blood cells were washed twice with buffer containing 150 mM NaCl, 25 mM glucose, 1 mM EDTA, 20 mM Tris at pH 7.4. Vesicles containing PC, PA and cholesterol (0.4:0.01:0.4 molar ratio) and trace amounts of [^{3}H]oleoyl-PC + [*choline*-^{14}C]PC were prepared as described by Kuypers et al. [15] and incubated with cells (hematocrit of 30) in the presence of 2–5 μM PC transfer protein at 37°C under mild agitation. The ratio of PC in the vesicles to PC in the erythrocyte membrane was 10. As the rate of exchange depends on the stage of infection [5] the length of the incubation period was chosen depending on the stage of infection. In control experiments the same amounts of vesicles and cells were incubated without transfer protein. After lipid extraction and analysis, the extent of PC transfer was calculated as described by Van Meer and Op den Kamp [16].

3. RESULTS

In a first approach, the PC specific transfer protein (PC-TP) was used to introduce [^{3}H,^{14}C]PC into the membrane of erythrocytes containing the schizont stage of the malaria parasite. Following this exchange step, we measured both the total amount of PC which had been transferred as well as the isotope ratio in the PC which was extracted from the erythrocytes (table 1).

The ^{3}H/^{14}C ratio of the PC used in the donor

Table 1

Introduction of phosphatidylcholine into erythrocytes

	PC transferred (%)	PC $^{3}H/^{14}C$ ratio
Control cells	31.1 ± 3	5.2 ± 0.3
Infected cells	174.0 ± 28	4.9 ± 1.0

[^{3}H]Oleoyl-[*choline*-^{14}C]PC was introduced during a 1 h incubation into control and schizont-infected erythrocytes using donor vesicles of PC, PA and cholesterol and the PC specific transfer protein (PC-TP). The ratio of $^{3}H/^{14}C$ in the vesicles was 6.1 ± 0.7. Incubations, carried out at 37°C, were performed with 60 μl of infected cells and 3 μM PC-TP in a total volume of 210 μl. Incubations with control cells were carried out with 250 μl of cells and 5 μM PC-TP in a volume of 750 μl. After incubation cells were washed and lipids were extracted, separated by TLC and analysed. Further details are described in section 2. The uptake data are expressed as % of the total PC present in the erythrocyte membranes and data are the mean values of three experiments

vesicles was 6.1 ± 0.7. After 1 h of incubation of control erythrocytes in the presence of these vesicles and the PC-TP, the PC extracted from the cells appeared to have an isotope ratio of 5.2 ± 0.3. The total extent of transfer amounted to 31.1 ± 3% of the PC present in the erythrocyte membrane. In the schizont infected cells the accumulation of radiolabeled PC was much higher and, when calculated on the basis of the erythrocyte PC present in the plasma membrane of infected erythrocytes, amounted to 174.0 ± 28%. The isotope ratio of this PC was not significantly lowered (4.9 ± 1) in comparison with control cells. No radioactivity was found either in other lipids or in the aqueous phase removed during lipid extraction. These results indicate that PC is introduced as an intact molecule into the infected erythrocyte and is not significantly subject to attack by phospholipases. The fact that more PC can be transferred than is present in the erythrocyte membrane of infected cells illustrates that also the PC of the parasite is available for transfer which is in agreement with previous observations [5]. It is furthermore obvious that the radiolabeled PC which is accumulated in the infected cells does not undergo any turnover during the 1 h incubation period.

In the above experiments, the introduction of radiolabeled PC into the erythrocyte membrane was facilitated by a PC specific transfer protein. With human control erythrocytes, this PC-TP has been shown to catalyze a one-for-one PC transfer without any net increase or decrease in the PC content of the cells. Infected cells, however, require large amounts of phospholipids for the synthesis of the parasite membranes and it cannot be excluded therefore that these cells have the capacity to accumulate phospholipid molecules directly from the medium without the need of a transfer protein. To investigate this possibility, similar incubations to those described above were carried out with trophozoite infected cells, in both the presence and absence of the PC-TP. In addition we have measured the isotope ratio of PC, 4 h after the introduction of this lipid into the infected cells. The results are summarized in table 2.

After 1 h incubation of infected cells in the presence of PC vesicles but without PC-TP an amount of PC, equivalent to 7.3% of the PC present in the erythrocyte membrane, was taken up by the cells. In a 5 h incubation period, the uptake increased to 61.8%. Previous experiments have clearly shown that under identical conditions the uptake of PC by uninfected cells is negligible. The isotope ratio of the accumulated PC was not changed in either of the two experiments whereas no radioactivity could be detected in other lipids or

Table 2

Introduction of phosphatidylcholine into trophozoite infected erythrocytes

Incubation time (h)		PC transferred (%)	PC $^{3}H/^{14}C$ ratio
+ PC-TP	− PC-TP		
0	1	7.3	2.50
0	5	61.8	2.50
1	0	36.2	2.41
5	0	187.2	2.89
1	4[a]	29.0	2.50

[^{3}H]Oleoyl-[*choline*-^{14}C]PC with a $^{3}H/^{14}C$ ratio of 2.68 was introduced using donor vesicles of PC, PA and cholesterol. Incubations, carried out at 37°C, were performed with 60 μl of infected cells, without the transfer protein as well as in the presence of 3 μM PC-TP, in a total volume of 210 μl. In one of the incubations PC-TP was present for 1 h only. Subsequent incubation for 4 h [a] was carried out in the absence of PC donor vesicles. After incubation cells were washed and lipids were extracted, separated by TLC and analysed. Further details are described in section 2. The uptake data are expressed as % of the total PC present in the erythrocyte membranes

in the water phase of the lipid extraction medium. The presence of PC-TP enhanced the transfer of radiolabeled PC towards the infected cell considerably and also in this case the isotope ratio of the PC remained unchanged (table 2). Even when PC is transferred to infected cells and the cells are subsequently incubated for 4 h in the absence of donor vesicles and transfer protein, the isotope ratio of the PC still remains unmodified.

These data clearly show that, in contrast to control cells, infected erythrocytes can accumulate intact PC molecules from the medium without the help of a transfer protein. Since previous experiments showed that the phospholipid content of the host membrane does not change during parasite growth it can be concluded that the newly introduced phospholipid is accumulated by the parasites. Neither the PC, which is acquired by exchange, nor the PC, which is directly taken up by the cells, appears to be metabolized to any appreciable extent.

3.1. *Uptake of PE and PS*

In similar experiments to those above, we have tried to investigate if the parasite can also make use of intact PE and PS molecules. To this end, infected cells and control erythrocytes were incubated with vesicles consisting of egg PC, egg PA and cholesterol and which also contained phosphatidyl[2-^{14}C]ethanolamine or phosphatidyl-[U-^{14}C]serine. Both the uptake of these components as well as their conversion into other lipids was studied. The non-specific lipid transfer protein from beef liver was added in a parallel series of experiments in order to facilitate the uptake of the radiolabeled phospholipids.

When uninfected cells are incubated with vesicles in the absence of non-specific lipid transfer protein less than 10% of the added vesicle material is recovered in the erythrocyte fraction and it is most likely that this represents non-specific adhesion of the vesicles to the cells, rather than actual uptake of the lipids into the erythrocytes. In the presence of non-specific lipid transfer protein however, the vesicle material is readily incorporated into the cells. The conditions were chosen such that the small amount of vesicle lipid added was taken up by the excess of erythrocytes within 30 min of incubation. Cells infected with *Plasmodium* at the trophozoite stage were able, in accordance with the experiments described above, to incorporate the vesicle phospholipids without the help of a transfer protein. Under similar conditions to those for the uninfected cells, all vesicle PS is incorporated in the infected cell in a 7 h incubation period, whereas in the presence of nsLTP complete uptake is achieved within 30 min.

The phosphatidylserine, which is taken up by uninfected cells, remains as such in the erythrocyte membrane; no radioactivity appeared in other lipids or in the water soluble fraction obtained during the lipid extraction (fig.1). In contrast, the PS, which is slowly incorporated into the trophozoite-infected cell, i.e. in the absence of transfer protein, is converted to a large extent into PE. Fig.1 shows that at the end of the incubation period, when all of the PS has been taken up by the cells, more than half of it is present as PE. In case the rate of PS uptake is accelerated by the presence of the transfer protein, the conversion of PS into PE appears to proceed at a slower rate.

Incubations carried out with vesicles containing PE instead of PS gave identical results with respect to the incorporation into the infected and control cells, with and without non-specific lipid transfer protein. The radiolabeled PE is converted into PC, be it that this conversion proceeds at a much lower rate than that of PS into PE. Furthermore, PC formation from radiolabeled PE is found only in those experiments in which the uptake of the PE is stimulated by the non-specific lipid transfer protein and does not affect the PE that is taken up in the absence of transfer protein (fig.2). It should be noted that, under the conditions applied here, the total amount of lipid which is taken up by the infected cell is rather small. The total phospholipid content of the erythrocyte membranes and intra-erythrocytic parasite membranes present amounts to about 90 nmol and 110 nmol, respectively, whereas 1.2 nmol of phospholipid are taken up at the end of the incubation period. The PS content, however, of the vesicles is relatively high (0.78 nmol) whereas PS is a minor constituent of the parasite membranes (1.7 nmol). This implies, assuming that all of the PS ends up in the parasite, that this organism receives an overload of this lipid. On the other hand, the PE content of the parasite is high (50 nmol) in comparison to the amount of PE introduced (0.5 nmol). Furthermore it should be realised in this respect that a major

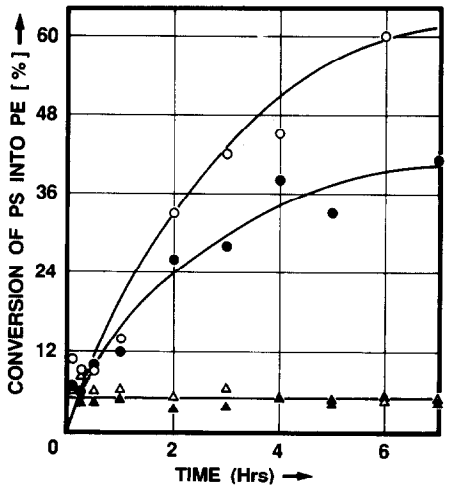

Fig.1. Conversion of PS into PE by intraerythrocytic trophozoites. [^{14}C]PS was transferred from PC/PA/PS/cholesterol vesicles (0.4:0.01:0.78:0.4 molar ratio) towards trophozoite-infected cells and uninfected cells, both in the presence and absence of the nsLTP. Incubations were carried out with 350 μl packed cells in a total volume of 1465 μl. After incubation the cells were washed, lipids were extracted, separated by TLC and analysed. The conversion of PS into PE is plotted as percentages of the total amount of PS which is incorporated into uninfected cells without nsLTP (△); into uninfected cells with nsLTP (▲); in infected cells without nsLTP (○) and in infected cells with nsLTP (●).

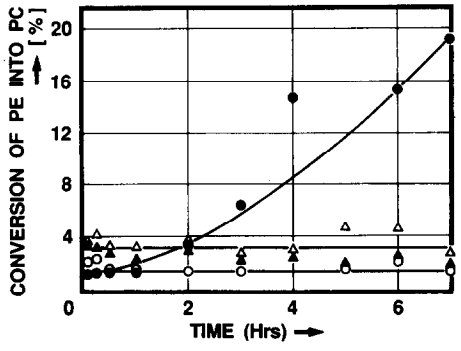

Fig.2. Conversion of PE into PC by intraerythrocytic trophozoites. [^{14}C]PE was transferred from PC/PA/PE/cholesterol vesicles (0.4:0.01:0.5:0.4 molar ratio) towards trophozoite-infected cells and control cells, both in the presence and absence of the nsLTP. Incubations were carried out with 350 μl packed cells in a total volume of 1550 μl. After incubation the cells were washed, lipids were extracted, separated by TLC and analysed. The conversion of PE into PC is plotted as percentages of the total amount of PE which is incorporated into uninfected cells without nsLTP (△); into uninfected cells with nsLTP (▲); in infected cells without nsLTP (○) and in infected cells with nsLTP (●).

constituent of the donor vesicles is PC, which is also transferred to the cells by the non-specific lipid transfer protein, and it is possible that accumulation of this phospholipid eliminates the need for PE conversion. The above mentioned data may have a role in the observed differences in the conversions of PS and PE.

4. DISCUSSION

The data presented here show that even in the absence of transfer protein uptake of PC, PE and PS by infected erythrocytes takes place. It is remarkable to note that infected cells have modified their membrane in such a way that passage of complex lipids is rapid. Based on the data presented by Taraschi et al. [18] on the increase in membrane fluidity, one may expect phospholipids to be translocated rapidly from the outer monolayer of the plasma membrane towards the inner monolayer. Whether, alternatively, crossing of the membrane by extracellular lipids might occur via pinocytosis or another invagination mechanism, by which large lipid structures could be taken up, is not known. Few data are available about transport systems within the infected erythrocyte. However it is obvious from the fact that more PC can be transferred than is present in the erythrocyte membrane that some kind of exchange or transport system must exist between the host membrane and the parasite. This can be a direct membrane to membrane contact (via the vacuolar membrane system?) but it is also possible that lipid transport proceeds via a vesicle system, as is observed in endocytotic and exocytotic processes, or it might be even mediated by a lipid transfer protein. A route has been described via which electron dense material is transported from the parasite through the cytoplasm towards the erythrocyte membrane [19]. Whether or not this represents a general pathway which can also be used in the opposite direction has to be investigated.

The data obtained with the double-labeled PC molecule strongly suggest that intact PC can be taken up and used as such for the biogenesis of parasite membranes. This implies that no appreciable phospholipase activity is exerted on PC molecules. Whether or not phospholipases are in-

volved in a release of fatty acids from other phospholipids requires elucidation.

The modification of the polar headgroups of PS and PE, which are observed in infected cells, once more suggests that these lipids have been incorporated into the parasite, since sufficient activity of PS decarboxylase and PE methylating enzymes is only present in the parasites. It is of interest to observe that both the rate and extent of headgroup modification differ for the PS and PE and that furthermore these processes depend on the way the lipids have been introduced. An explanation of this phenomenon is not readily available, especially because the mechanism of action of the non-specific lipid transfer protein, the final localization of the newly introduced phospholipids and the localization, within the parasite, of the enzymes involved are unknown. The difference between the protein mediated transfer and the incorporation of phospholipids in the absence of a protein could be that phospholipids end up in different pools or membrane systems. That the procedure by which phospholipids are introduced into a cell may affect the final fate of those lipids has been shown before in studies with Friend erythroleukemic cells [20]. Phosphatidylinositol (PI) introduced into these cells with the non-specific transfer protein is, in this case, relatively less susceptible to metabolic conversions than the PI which is introduced without this transfer protein. Further experimentation will be necessary to clarify these observations.

In summary, we demonstrate in this paper that intact PC, PE and PS can migrate from vesicles towards the intraerythrocytic malaria parasite. Further investigation needs to quantify the importance in vivo of the transport of intact extracellular lipids towards the malaria parasite.

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