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Plasmodium knowlesi induces alterations in phosphatidylcholine and phosphatidylethanolamine molecular species composition of parasitized monkey erythrocytes

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Using high performance liquid chromatography and gas-liquid chromatography, we have characterized the phosphatidylcholine and phosphatidylethanolamine molecular species composition of trophozoite and schizont forms of *Plasmodium knowlesi* parasitized erythrocytes. Similarly, we determined these parameters in the erythrocyte membranes of trophozoite parasitized cells, unparasitized erythrocytes from infected monkeys before and after a chloroquine treatment and erythrocytes from monkeys that had never been infected. Plasma phosphatidylcholine molecular species composition was also studied. *P. knowlesi* parasitized erythrocytes presented higher amounts of 16:0/18:2-phosphatidylcholine than the various control cells, which appeared to be compensated for by a decrease in 18:0/20:4-, 16:0/20:3-, 16:0/18:1-, 18:0/18:2-, 18:0/20:3-, 16:0/16:0- and 16:0/18:0-phosphatidylcholines. In the case of phosphatidylethanolamine, the alterations were quantitatively of greater importance and consisted of an increase in, again, 16:0/18:2-phosphatidylethanolamine and a decrease in several species containing 20:4, namely 16:0/20:4-, 18:0/20:4- and 18:1/20:4-phosphatidylethanolamine; also the levels of alkoxy-phosphatidylethanolamines were markedly decreased. *P. knowlesi* development within monkey erythrocytes therefore appears to be associated with changes in phosphatidylcholine and phosphatidylethanolamine molecular species in the whole parasitized cell. These alterations are also exhibited by the host cell membrane, which provides the first experimental evidence that the parasite is able to manipulate the erythrocyte membrane lipid species composition. The consequences of these alterations on membrane physiology are discussed, as well as the implications that these data may have on the trafficking of phosphatidylcholine and phosphatidylethanolamine in the erythrocytes of *P. knowlesi* infected monkeys.

Introduction

During the intraerythrocytic development of the malaria parasite, the host cell membrane undergoes several changes in its biochemical and biophysical properties [1]. There are several indications pointing towards an increase in fluidity within the lipid bilayer [2–4], which might be related to the increase in its permeability [1,5,6]. It is worth mentioning that the high amounts of nutrients that are required by the intraerythrocytic parasite, are present in the plasma and, therefore, in order to became available to the Plasmodium, have to cross the host cell membrane.

Several parameters, such as the protein to lipid and cholesterol to phospholipid ratios, lipid classes composition, degree of unsaturation of the fatty acids and the relative position of the fatty acids at the glycerol backbone, i.e., phospholipids molecular species composition, are capable of influencing the fluidity within the lipid bilayer [8,9]. During Plasmodium intraerythrocytic development, several proteins of parasitic origin are inserted into the host cell membrane [10,11], which is likely to result in an increase of the overall protein to lipid ratio. Plasmatic cholesterol levels in malaria infected monkeys are higher than in healthy ones [12],

Abbreviations: CPT, cholinephosphotransferase; DPH, 1,6-diphenyl-1,3,5-hexatriene; Hepes, 2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid; HPLC, high performance liquid chromatography; HPTLC, high performance thin layer chromatography; GLC, gas-liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TLC, thin-layer chromatography.

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and also the cholesterol present in the plasma membrane of both parasitized and unparasitized erythrocytes seems to be increased accordingly [13]. Since both an increase in protein/lipid and cholesterol/phospholipid ratios usually have a rigidifying effect on biomembranes [8], these factors are not likely to be responsible for the observed increase in fluidity of the host cell membrane of parasitized erythrocytes. The total degree of unsaturation of the fatty acids from whole parasitized erythrocytes [14] does not change upon infection and neither does the lipid classes composition of the host erythrocytes membrane [15]. Therefore, it seemed of interest to investigate whether its phospholipid molecular species composition might change upon *P. knowlesi* development.

In this work we report several alterations in the phosphatidylcholine (PC) and phosphatidylethanolamine (PE) molecular species composition of monkey erythrocytes to occur during the development of *P. knowlesi* and which appear to be manifest also in the host cell membrane. Furthermore, the plasma PC molecular species composition of healthy and malaria-infected monkeys was studied. Taken as a whole, these data are also relevant for understanding the processes of renewal/acquisition of PC in *P. knowlesi* parasitized erythrocytes.

Materials and Methods

Chemicals

Acetic acid, ammonia solution (25%), benzene, boron trifluoridemethanol complex (20% solution in methanol), diethyl ether, 4-dimethylaminopyridine, ethanol, Giemsa' solution, hexane, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (Hepes), methanol, 2-propanol, were purchased from Merck. Benzoic anhydride and 1,6-diphenyl-1,3,5-hexatriene (DPH) were from Janssen Chimica. Acetonitrile was from J.T. Baker. Dichloro (*R*)fluorescein was from BDH. Percoll was from Pharmacia. RPMI 1640 was from GIBCO. Phospholipase C from *Clostridium welchii* was purified from a commercial preparation (Sigma, St. Louis, MO, U.S.A.) following the procedure described by Zwaal et al. [16] and phospholipase C from *Bacillus cereus* was purified according to Little [17].

Boron trifluoride-methanol complex (20% solution in methanol), was of synthesis grade; hexane, 2-propanol and acetonitrile were of chromatography grade; all the other chemicals were of pro analysis grade.

Isolation of cells and plasma

Rhesus monkeys (*Macaca mulatta*) and cynomolgus monkeys (*Macaca fascicularis*) were obtained from Shamrock Farm Henfield, Sussex, U.K. and Sanofi Research Centre, Montpellier, France, respectively. They were fed ad libitum with artificial aliment (No. 107 from UAR, Epinay sur Orge, France).

All animals had been splenectomized and were susceptible to infection after four weeks of convalescence. They were cured by an intramuscular injection of 150 mg chloroquine on three consecutive days; after six weeks the monkeys were eventually reinfected. *Plasmodium knowlesi* (Washington strain, variant 1, Dr. G. Mitchell, Guy's Hospital Medical School, London, U.K.) was used to infect the monkeys through an intravenous inoculation with $(2–6) \cdot 10^8$ parasitized cells, previously cryopreserved [18], or from monkey to monkey. Parasite intraerythrocytic development was monitored by determining the parasitemia (percentage of total erythrocytes that were infected) in daily blood smears coloured with Giemsa' solution. Blood was aseptically removed from each monkey by venipuncture into citric acid/dextrose after the monkey had been anaesthetized by an intramuscular injection of ketamine (IFFA-Credo, France). Blood was drawn from infected monkeys when the parasitemia was 10–30%. The never infected monkeys had been in the animal house for at least two months, when the blood was drawn from them. After centrifugation for 15 min at $700 \times g$, the plasma was removed and the cell suspended in RPMI 1640 supplemented with 25 mM Hepes (pH 7.4). White cells were removed by passage through a cellulose powder column CF 11 (Whatman) [19]. Unparasitized and parasitized erythrocytes were separated on a Percoll gradient [20], which allows the recovery of the parasitized cells in a fraction with 95–100% parasitemia. In order to obtain a pure host cell plasma membrane from *P. knowlesi* parasitized erythrocytes, polycationic microbeads (Affigel 731, Bio-Rad) were used [21]. In brief, after attaching parasitized erythrocytes to the microbeads, the cells were lyzed with hyposmotic buffer, 10 mM $NaPO_4$ (pH 7.1), and vigorous vortexing, followed by sonication in an ice-cold bath sonifier (Bransonic 221). The beads were allowed to sediment and the supernatant was collected. After repeating the process, the two supernatants were added together and the lipids from both the membranes attached to the beads and the supernatant were extracted. Parasitic contamination in the host cell plasma membrane fraction obtained by this method is lower than 5% [21]. Since 9–16% of the initial host cell membranes is attached to the beads [21], the supernatant fraction, referred to throughout the text as whole parasitized erythrocytes, is slightly enriched in parasites. It should be kept in mind that the lipids extracted from whole parasitized erythrocytes are derived from both the host erythrocyte membrane and the parasite. The relative amounts of these two components depend on the stage of parasite development. The phospholipid present in the the host cell membrane corresponds to 20–40% and 15–20% of the total, in the trophozoite and schizont stages, respectively [15].

Phosphatidylcholine and phosphatidylethanolamine isolation

Lipids from the different fractions were extracted according to the following methods that, in each case and in our conditions, gave a complete recovery of the two phospholipid classes concerned, i.e., PC and PE. Lipids from the various unparasitized erythrocyte fractions were extracted according to Rose and Oklander [22]; lipids from the membranes attached to the microbeads and from the plasma were extracted following a modified Folch procedure [23]; and lipids from whole parasitized erythrocytes were extracted as described by Bligh and Dyer [24]. All lipids were stored in chloroform/methanol (2:1, v/v) at −20°C under nitrogen. PC and PE were separated from the other lipids by a two-dimensional TLC (Pre-coated TLC plates, Silica Gel 60, Merck) using as solvent systems chloroform/methanol/ammonia 25% (v/v)/water (90:54:5.5:5.5, v/v) and chloroform/methanol/acetic acid/water (90:40:12:2, v/v) [25]. The lipid spots were visualized under ultraviolet light after spraying the TLC plates with 0.03% DPH in chloroform. PC and PE were scraped off and extracted from the silica [26].

Phosphatidylcholine and phosphatidylethanolamine molecular species analysis

PC and PE were converted into diradylglycerobenzoates for HPLC analysis as described by Blank et al. [27]. In brief, PC and PE were submitted to a *C. welchii* phospholipase C (0.5 IU/300 nmol of PC) [28] and a *Bacillus cereus* phospholipase C (1 IU/300 nmol of PE) catalyzed hydrolysis [29], respectively. The resulting diradylglycerols were converted into diradylglycerobenzoate derivatives by dissolving them in 0.3 ml of benzene containing 10 mg of benzoic anhydride and 4 mg of 4-dimethylaminopyridine, and allowing the mixture to stand for 1 h at room temperature. The reaction was stopped by slowly adding 2 ml of 0.1 M NaOH, while vortexing. The diradylglycerobenzoates were then separated into subclasses, namely 1-alkenyl-2-acyl, 1-alkyl-2-acyl and diacyl species, on HPTLC plates (Precoated HPTLC plates, Silica Gel 60, Merck), using as solvent system benzene/hexane/diethyl ether (50:45:5, v/v). The lipid spots were visualized under ultraviolet after spraying the plate with dichlorofluorescein. Subclasses were scraped off and extracted from the silica with 2 ml 96% ethanol; after adding 2 ml distilled water, the lipids were recovered by washing the aqueous solution three-times with 2 ml hexane. Separation of molecular species within each diradylglycerobenzoate subclass was accomplished by isocratic elution with acetonitrile/2-propanol (70:30, v/v), using a Gilson HPLC system fitted with a 4.6 × 250 mm analytical column packed with 5 μm Ultrasphere ODS (Altex), operated at a flow rate of 1 ml/min. The separated components were detected at 230 nm with a Pye Unicam LC_3 Ultraviolet detector and integrated on-line with the help of a Gilson 621 Data Master Module coupled computer. Individual fractions were identified by comparing their retention times with those of standards in known mixtures analyzed under identical conditions [27]. The total amount of each subclass of diradylglycerobenzoates was quantified from the total HPLC integration units [27]. In the case of co-migrating molecular species, the quantification of individual components was done by collecting the corresponding HPLC eluent and analysing the lipids by GLC. Due to a shortage of material, the peaks of multiple samples of the same type had to be pooled together (this made it impossible to calculate S.D. values of these data). The technique used here does not allow us to discriminate between the two isomers of a particular molecular species, which differ from each other with respect to the localization of their two aliphatic chains at the 1- and 2-positions of the glycerol backbone [27]. It is known, however, that unsaturated acylchains are preferentially located at the 2-position, while the majority of the saturated fatty acids are found at the 1-position of the glycerol moiety [30]. Therefore, the molecular species will be described by first indicating the acyl chain that is more likely to be esterified at the glycerol 1-position.

Fatty acid analysis

Fatty acid methyl esters were prepared from HPLC fractions by the boron-fluoride method [31] and separated on a Perkin-Elmer 8500 Capillary Gas Chromatograph equipped with a 50m × 0.26mm WCOT Fused Silica CP-Sil-88 column (Chrompack). The temperature of the split injector and the Flame Ionization Detector was 270°C; the initial oven temperature of 180°C was kept constant for 6 min, then heated up to 200°C at 20 C°/min, maintained at 200°C for 5 min, again heated up to 230°C at 20 C°/min and finally kept at this temperature for 11.5 min. Identification of the different components was done by comparing their retention times with those of known standards.

Results

Regarding PC, the amounts of 1-alkenyl-2-acyl and 1-alkyl-2-acyl subclasses accounted for less than, respectively, 1.5% and 0.5% of the total PC in all the samples. This fact, together with the limited amount of available biological material, precluded a detailed analysis of molecular species in these subclasses.

Table I shows the main diacyl molecular species of PC found in several fractions isolated from *P. knowlesi* infected monkeys, namely: unparasitized erythrocytes, the host cell membrane of parasitized erythrocytes and whole parasitized erythrocytes at two different stages of parasite development, the trophozoite and the schizont. At the trophozoite stage, the parasite is differentiated in

TABLE I

Phosphatidylcholine diacyl species composition (mol%) in erythrocyte membranes of unparasitized and trophozoite parasitized monkey cells, and in trophozoite and schizont forms of P. knowlesi parasitized erythrocytes

Diacylglycerobenzoate molecular species obtained from PC, following the procedure described under Materials and Methods, were separated by HPLC. The amount of each molecular species within multispecies HPLC peaks was determined by GLC. HPLC data are presented as means ± S.D. and the number of independent determinations (n) is indicated; HPLC fractions corresponding to <1% of the total have been omitted. Data derived by GLC are presented between brackets; n.d., not detectable. Statistically significant differences, determined by the Student's t-test, between the unparasitized erythrocytes and each of the other types of samples are indicated: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$. Although not shown in the table, statistically significant differences between trophozoite and schizont data were found, namely in peaks A ($P < 0.005$), E ($P < 0.01$), F ($P < 0.005$) and G ($P < 0.05$).

HPLC peak	PC molecular species	Erythrocyte plasma membrane		Parasitized erythrocytes	
		unparasitized cells ($n = 9$)	trophozoite parasitized cells ($n = 3$)	trophozoite ($n = 3$)	schizont ($n = 3$)
A	16:0/22:6	2.7±0.4	2.4±1.1	4.1±0.2 ***	2.6±0.4
B	18:1/20:4	0.8±0.3	1.0±0.1	1.4±0.2 **	1.2±0.7
C	16:0/20:4	5.3±1.1	4.2±1.3	5.4±0.3	5.6±0.3
D	18:1/18:2	4.3±0.4 (2.6)	4.0±0.1 (3.5)	4.9±0.4 (4.9)	4.5±0.4 (4.1)
	18:1/20:3	(1.6)	(0.5)	(n.d.)	(0.4)
E	16:0/18:2	40.2±3.7 (36.3)	49.0±3.5 *** (44.9)	48.7±2.0 *** (44.1)	55.5±0.7 *** (50.5)
	16:0/20:3	(3.9)	(2.9)	(3.3)	(3.2)
	16:0/22:4	(n.d.)	(1.2)	(1.3)	(1.8)
F	18:0/20:4	5.0±1.9 (3.6)	1.6±0.7 ** (1.6)	2.4±0.1 (2.4)	1.1±0.0 *** (1.1)
	16:0/20:3	(1.4)	(n.d.)	(n.d.)	(n.d.)
G	16:0/18:1	30.6±1.9 (10.9)	28.1±3.9 (10.0)	25.6±0.7 *** (11.8)	23.4±1.0 *** (10.3)
	18:0/18:2	(17.7)	(16.0)	(12.1)	(11.7)
	18:0/20:3	(2.1)	(1.9)	(1.2)	(1.1)
	18:0/22:4	(n.d.)	(0.2)	(0.5)	(0.5)
H	16:0/16:0	7.0±2.1	5.1±0.6	3.3±0.1 **	2.9±0.5 ***
I	18:0/18:1	2.1±0.4 (1.8)	3.1±1.1 (1.8)	3.1±0.2 *** (2.5)	2.3±0.1 (1.8)
	16:0/20:1	(0.3)	(1.4)	(0.5)	(0.5)
J	16:0/18:0	1.9±0.2	1.6±0.4	1.1±0.3 ***	<1

an uninucleate amoeboid cell which, in time, undergoes nuclear division thereby leading to the appearance of the schizont stage [1]. The data referring to unparasitized erythrocytes have been derived from blood from five monkeys, showing 20–30% parasitemia at different stages of development of the parasite. No differences could be observed between the PC molecular species composition in unparasitized cells originating from the various monkeys (data not shown). In an attempt to discern the PC molecular species composition of the host cell membrane from that of the parasite, PC obtained from the host cell membrane and PC from whole parasitized erythrocytes from the same monkey (Table I) were analyzed. This monkey showed a 20% parasitemia and approx. 70% of the parasite population consisted of trophozoites, the remaining 30% being constituted by both earlier and later (schizont) stages. The PC molecular species in whole schizont parasitized erythrocytes was also determined (Table I), using cells obtained from a monkey that showed a 30% parasitemia and an apparently homogeneous schizont population. In each of the four types of samples, there is a notorious predominance of species with a saturated and an unsaturated acylchain (mixed species); they constitute more than 80% of the total PC molecular species. It is worth noting that the disaturated species, namely 16:0/16:0 and 16:0/18:0, are decreased in trophozoite and schizont parasitized erythrocytes. The diunsaturated species, 18:1/20:4, 18:1/18:2, and 18:0/20:3, on the other hand, do not show any consistent variation within the four types of samples. As far as the mixed species are concerned, there is a striking increase in 16:0/18:2 which is associated with *P. knowlesi* development. This change is accompanied by a decrease in the 18:0/18:2, 18:0/20:3 and 18:0/20:4 species. If one compares the PC diacyl species found in the host cell plasma membrane of trophozoite parasitized erythrocytes with those found in whole parasitized erythrocytes, the only statistically significant differences are found in 16:0/16:0, which is decreased in the parasite

($P < 0.005$), and in 16:0/22:6, which is increased ($P < 0.05$). On the other hand, the two different stages of parasite development which have been studied, i.e., trophozoite and schizont, show several differences regarding their PC molecular species composition. The development of the trophozoite into a schizont is associated with a further increase in 16:0/18:2.

Table I provides an overview of the PC molecular species composition of *P. knowlesi* infected monkey erythrocytes. However, to gain further insight into the extent by which these alterations are associated with the disease, these data should be considered in view of the situation in the red cell of the healthy monkey. As becomes clear from the data shown in Table II, some differences in PC molecular species composition are also observed in the membranes of unparasitized cells from an infected monkey, when compared to that in the red cell membrane of a never infected animal. The most striking difference again concerns an increase in 16:0/18-2-PC (Table II). It furthermore seemed of interest to investigate whether the *P. knowlesi* induced alterations in the PC molecular species composition in unparasitized erythrocytes are maintained following a successful chloroquine treatment. Interestingly, the alterations in PC molecular species composition of unparasitized erythrocytes do not completely disappear after a chloroquine treatment. The PC molecular species composition in the case of chloroquine treated monkeys was determined in blood samples from two monkeys and, although in one case the blood was drawn immediately after the treatment and in the other case 19 days thereafter, no difference could be observed (data not shown). For comparative reasons, a species composition analysis of PC from plasma of healthy and malaria-infected monkeys (12–25% of parasitemia, mainly trophozoites) was carried out. Only slight differences could be observed, although an increase in 16:0/18:2-PC might eventually be related with a similar one occurring in the unparasitized erythrocytes of infected monkeys (see below). The plasma PC molecular species composition resembles that of the various control erythrocytes as far as the main species are concerned. However, the levels of disaturated species are lower, while the levels of 16:0/20:4, 18:1/18:2 and 18:1/20:3 are higher in plasma than in the erythrocytes.

The results presented in both Tables I and II show that *P. knowlesi* infection of a monkey is associated with a marked increase in the 16:0/18:2 and a decrease

TABLE II

Phosphatidylcholine diacyl species composition (mol%) in unparasitized erythrocytes obtained from never infected and P. knowlesi infected monkeys before and after a chloroquine treatment, and in monkey blood plasma

The data were obtained and are presented as described in the legend of Table I. Statistically significant differences between erythrocytes from never infected and infected monkeys, as well as between plasma from healthy and infected monkeys are indicated: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$.

HPLC peak	PC molecular species	Erythrocytes from			Plasma from	
		never infected monkeys ($n = 3$)	*P. knowlesi* infected monkeys		healthy monkeys ($n = 6$)	infected monkeys ($n = 6$)
			before chloroquine ($n = 9$)	after chloroquine ($n = 3$)		
A	16:0/22:6	3.9±0.5	2.7±0.4 **	2.6±0.6 ***	5.3±0.5	3.1±0.8 ***
B	18:1/20:4	1.0±0.2	0.8±0.3	1.1±0.2	1.3±0.4	1.3±0.2
C	16:0/20:4	4.9±0.5	5.3±1.1	6.2±0.6 ***	7.1±1.2	6.2±1.2
D	18:1/18:2	4.5±0.7 (4.5)	4.3±0.4 (2.6)	4.2±0.2 (2.6)	7.1±0.8 (6.3)	5.9±2.0 (5.6)
	18:1/20:3	(n.d.)	(1.6)		(0.7)	(0.3)
E	16:0/18:2	33.3±3.2 (28.3)	40.2±3.7 * (36.3)	38.5±2.4 * (32.2)	34.3±1.7 (29.8)	36.4±2.1 * (33.2)
	16:0/20:3	(5.0)	(3.9)	(4.1)	(3.8)	(2.8)
	16:0/22:4	(n.d.)	(n.d.)	(1.7)	(0.7)	(0.2)
F	18:0/20:4	5.6±0.5 (4.1)	5.0±1.9 (3.6)	5.6±0.8 (4.5)	7.5±1.1 (5.5)	6.7±1.3 (6.6)
	16:0/20:3	(1.5)	(1.4)	(1.2)	(2.0)	(0.1)
G	16:0/18:1	33.2±0.2 (14.1)	30.6±1.9 *** (10.9)	30.0±0.4 *** (14.7)	33.8±1.4 (10.5)	35.2±1.1 (9.3)
	18:0/18:2	(15.6)	(17.7)	(12.1)	(19.3)	(23.8)
	18:0/20:3	(3.4)	(2.1)	(2.1)	(3.2)	(2.1)
	18:0/22:4	(n.d.)	(n.d.)	(n.d.)	(0.8)	(n.d.)
H	16:0/16:0	9.2±0.6	7.0±2.1	5.9±0.9 ***	1.0±0.1	2.6±0.8 ***
I	18:0/18:1	2.6±0.9 (2.6)	2.1±0.4 (1.8)	3.7±0.9 (2.6)	2.6±0.8 (2.6)	2.8±0.7 (2.8)
	16:0/20:1	(n.d.)	(0.3)	(0.4)	(n.d.)	(n.d.)
J	16:0/18:0	1.8±0.3	1.9±0.2	2.1±0.4	<1	<1

in the 18:0/20:4, 16:0/20:3, 16:0/18:1, 18:0/18:2, 18:0/20:3, 16:0/16:0 and 16:0/18:0 species of their red cell PC. An increase in some minor species, namely 16:0/22:4, 18:0/22:4 and 16:0/20:1, can also be noted. The differences in PC molecular species between the control, i.e., never infected monkey erythrocytes and the other samples, increase according to the following order: erythrocytes from chloroquine treated monkeys ≅ unparasitized erythrocytes from infected monkeys < host cell membranes from trophozoite parasitized erythrocytes ≅ trophozoite-parasitized erythrocytes < schizont-parasitized erythrocytes. It is worth noting that these alterations have no apparent consequences as to the unsaturation index, defined as the mean number of double bonds per acyl chain. This index has a value of 1.09 ± 0.06, considering the total fatty acid composition as can be calculated from the data presented in Tables I and II, and considering all the types of cells together.

Unparasitized erythrocytes, both from never infected and from *P. knowlesi* infected monkeys, appeared to contain appreciable amounts of 1-alkyl-2-acyl- and 1-alkenyl-2-acyl-PE, allowing an accurate HPLC analysis. In erythrocytes from never infected monkeys ($n = 3$) these subclasses comprised, respectively, 15.6 ± 1.0 and $8.3 \pm 1.4\%$ from the total PE species, whereas in unparasitized erythrocytes from *P. knowlesi* infected monkeys ($n = 4$) they accounted for 14.9 ± 5.1 and $5.4 \pm 1.5\%$, respectively. The HPLC species analysis revealed no differences between the two sets of samples (data not shown). *P. knowlesi* intraerythrocytic development was associated with a marked decrease in the relative amount of both 1-alkyl-2-acyl- and 1-alkenyl-2-acyl-PE, including the fractions in the host cell membrane of trophozoite parasitized erythrocytes. This decrease, which amounted to 70–90%, together with the reduced amounts of available biological material, precluded a satisfactory integration of the HPLC elution profile. The decrease in plasmalogens within PE (1-alkenyl-2-acyl-PE) of whole *P. knowlesi* parasitized erythrocytes has been previously reported in a work where GLC was used [14].

Table III shows the main diacyl molecular species

TABLE III

Phosphatidylethanolamine diacyl species composition (mol%) in erythrocyte membranes of unparasitized and trophozoite parasitized monkey cells, and in trophozoite and schizont forms of P. knowlesi parasitized erythrocytes

Diacylglycerobenzoate molecular species obtained from PE, following the procedure described under Materials and Methods, were separated by HPLC. The amount of each molecular species within multispecies HPLC peaks was determined by GLC. HPLC data are presented as means ± S.D. and the number of independent determinations (n) is indicated; HPLC fractions corresponding to <1% of the total have been omitted. Data derived by GLC are presented between brackets; n.d., not detectable. Statistically significant differences, determined by the Student's t-test, between the unparasitized erythrocytes and each of the two other types of samples are indicated: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$. Although not shown in the Table, statistically significant differences were found between trophozoite and schizont data (peak A, $P < 0.005$), and between host cell membrane and entire parasitized cell in trophozoite parasitized erythrocyte (peaks B, $P < 0.005$; I, $P < 0.05$; and J, $P < 0.05$).

HPLC peak	PE molecular species	Erythrocyte plasma membrane		Parasitized erythrocytes	
		unparasitized cells ($n = 5$)	trophozoite parasitized cells ($n = 3$)	trophozoite ($n = 3$)	schizont ($n = 3$)
A	16:0/22:6	11.2 ± 0.7	6.2 ± 1.2 ***	7.7 ± 0.0 ***	10.1 ± 0.7
B	18:1/20:4	7.2 ± 1.1	1.6 ± 0.3 ***	2.6 ± 0.1 ***	2.7 ± 0.7 ***
C	16:0/20:4	20.7 ± 1.6	5.7 ± 0.3 ***	5.5 ± 0.3 ***	5.0 ± 0.6 ***
D	18:1/18:2	10.2 ± 0.4 (7.7)	12.1 ± 0.4 *** (10.7)	12.4 ± 0.1 *** (11.1)	13.1 ± 0.9 *** (11.8)
	18:1/20:3	(0.9)	(0.8)	(0.7)	(0.7)
	18:1/22:4	(1.6)	(0.6)	(0.6)	(0.6)
E	16:0/18:2	23.3 ± 2.0 (17.6)	43.0 ± 1.8 *** (38.3)	41.3 ± 0.6 *** (36.0)	43.1 ± 1.7 *** (37.5)
	16:0/20:3	(2.8)	(2.3)	(2.6)	(2.4)
	16:0/22:4	(3.0)	(2.4)	(2.7)	(2.8)
F	18:0/20:4	9.9 ± 0.9 (9.5)	2.1 ± 0.7 *** (1.2)	2.1 ± 0.4 *** (1.9)	1.6 ± 0.7 *** (1.4)
	16:0/20:3	(0.4)	(0.9)	(0.2)	(0.2)
G	16:0/18:1	14.4 ± 1.0 (6.3)	20.4 ± 1.0 *** (11.0)	21.2 ± 2.0 *** (10.6)	19.1 ± 1.3 *** (9.5)
	18:0/18:2	(5.6)	(7.6)	(9.2)	(8.9)
	18:0/20:3	(1.0)	(0.8)	(0.7)	(0.6)
	18:0/22:4	(1.4)	(1.1)	(0.8)	(0.7)
H	16:0/16:0	<1	2.0 ± 0.4	2.3 ± 0.9	2.1 ± 0.7
I	18:0/18:1	2.1 ± 0.5	5.3 ± 0.6	3.5 ± 1.0 *	2.1 ± 0.0
J	16:0/18:0	<1	1.8 ± 0.2	1.3 ± 0.2	1.1 ± 0.1

composition of PE found in the above mentioned fractions isolated from *P. knowlesi* infected monkeys. In each of the four sets of samples there is again a predominance of mixed species, which constitute more than 80% of the total PE diacyl species. However, if one compares the unparasitized erythrocytes data for PE with those obtained from the same cells for PC, it becomes clear that the two lipid classes exhibit quite distinct species composition. In particular the 16:0/18:1, 16:0/18:2 and 18:0/18:2-species, which together represent 65% of the total diacyl PC species, constitute only 33% of the total diacyl PE. On the other hand, species with 20:4 represent a more considerable portion of PE diacyl species when compared to those of PC (37 and 10%, respectively). The existence of marked differences between unparasitized and *P. knowlesi* parasitized erythrocytes is evident (Table III). All the species containing 20:4, namely 16:0/20:4, 18:0/20:4 and 18:1/20:4, are severely decreased in the three groups of samples obtained from parasitized erythrocytes. Concomitantly, there is a marked increase in 16:0/18:2 and smaller increases in 16:0/18:1 and 16:0/16:0. If one compares the PE diacyl species composition of the host cell plasma membrane of trophozoite-parasitized erythrocytes with that of whole trophozoite-parasitized erythrocytes, they appear to be essentially identical. The same applies, grosso modo, to the PE diacyl species composition of trophozoite and schizont-parasitized cells, where the only statistically significant difference was found in 16:0/22:6 ($P < 0.05$). The diacyl PE fraction from parasitized cells shows lower unsaturation indexes (1.2 and 1.4 for trophozoite and schizont parasitized cells, respectively) than the corresponding fraction from unparasitized erythrocytes (1.8).

The range of variations of PE diacyl molecular species composition among unparasitized cells isolated from never infected monkeys and those from animals before and after a chloroquine treatment, is very narrow (Table IV). Furthermore, comparing erythrocytes from never infected monkeys with the unparasitized ones from infected monkeys, the alterations occurring in the diacyl PE molecular species composition in the parasitized cells seem to be mimicked only in two cases, namely the decrease in 16:0/22:6- and the increase in 16:0/18:2-PE. Taken together, the data presented in Tables III and IV reveal that intraerythrocytic development of *P.*

TABLE IV

Phosphatidylethanolamine diacyl species composition (mol%) in unparasitized erythrocytes obtained from never infected and P. knowlesi infected monkeys before and after a chloroquine treatment

The data were obtained and are presented as described in the legend of Table IV. Statistically significant differences with corresponding values in the samples from the never infected monkeys are indicated: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$. Although not shown in the table, statistically significant differences between unparasitized erythrocytes obtained before and after a chloroquine treatment were found, namely in peaks A ($P < 0.005$), D ($P < 0.005$), E ($P < 0.05$) and F ($P < 0.005$).

HPLC peak	PE molecular species	Never infected ($n = 3$)	*P. knowlesi* infected	
			before chloroquine ($n = 5$)	after chloroquine ($n = 3$)
A	16:0/22:6	13.7±0.6	11.2±0.7 ***	10.3±0.2 **
B	18:1/20:4	8.1±0.7	7.2±1.1	7.9±0.1
C	16:0/20:4	19.9±0.7	20.7±1.6	22.6±1.1 *
D	18:1/18:2	9.5±0.1 (7.7)	10.2±0.4 * (7.7)	8.5±0.7 (6.4)
	18:1/20:3	(0.8)	(0.9)	(1.2)
	18:1/22:4	(1.0)	(1.6)	(0.9)
E	16:0/18:2	19.3±1.4 (16.3)	23.3±2.0 *(17.6)	20.1±0.3 (14.0)
	16:0/20:3	(1.0)	(2.8)	(2.9)
	16:0/22:4	(2.0)	(3.0)	(3.2)
F	18:0/20:4	12.3±1.5 (11.8)	9.9±0.9 * (9.5)	15.0±0.4 * (14.0)
	16:0/20:3	(0.5)	(0.4)	(1.0)
G	16:0/18:1	14.9±0.6 (5.8)	14.4±1.0 (6.3)	13.5±2.0 (4.8)
	18:9/18:2	(7.0)	(5.6)	(6.3)
	18:0/20:3	(0.9)	(1.0)	(1.0
	18:0/22:4	(1.2)	(1.4)	(1.6)
H	16:0/16:0	<1	<1	<1
I	18:0/18:1	1.6±0.3	2.1±0.5	1.7±0.2
J	16:0/18:0	<1	<1	<1

knowlesi results in gross changes of PE molecular species composition of the parasitized cells. These changes, which seem to affect only the parasitized erythrocytes, are likely to occur at an early stage of parasite development, since trophozoite and schizont parasitized erythrocytes exhibit a similar PE molecular species composition. At least in the trophozoite stage, they also affect the host cell membrane, because its composition is very similar to that of the entire parasitized erythrocyte. As far as PE molecular species are concerned, the main differences between unparasitized and *P. knowlesi* parasitized erythrocytes seem to consist of a marked decrease in 1-alkyl-2-acyl and 1-alkenyl-2-acyl subclasses and, within the diacyl subclass, a decrease in 16:0/20:4-, 18:0/20:4- and 18:1/20:4- and an increase in 16:0/18:2-PE.

The overall fatty acid composition of PC and PE from healthy and entire *P. knowlesi* infected erythrocytes, as determined by direct GLC analysis, has been reported previously [14]. Such data can also be calculated from the molecular species composition as determined in this study and, when doing so (not shown), they appear to be in fair agreement with those previously reported, be it that 22:4 and 22:6 had not been detected before in the PC fraction. In order to confirm our data which showed the presence of 22:4 and 22:6 in the molecular species (in HPLC fractions A, E and G) of PC, we analyzed also the overall fatty acid composition of the total PC and indeed were able to detect these fatty acids. At present the actual reason for this difference is not known.

Discussion

Our results show that *P. knowlesi* is able to induce drastic changes in the erythrocytic PC and PE molecular specific composition, in spite of its notorious inability to synthesize fatty acids de novo [32]. At least in the trophozoite stage, where there is an extensive lipid enrichment of the parasitized cell accompanying the full development of the parasite and preceding its schizogony [1], the alterations found in the lipids of entire parasitized erythrocytes are also detectable in the host cell membrane. Considering that PC plus PE together comprise 60–70% of the total phospholipid of this membrane [15], this observation indicates that an extensive exchange of lipids between parasite and host erythrocyte must occur. Furthermore, it means that the intraerythrocytic parasite is able to induce marked changes in the host cell membrane, from which it is separated by the erythrocyte cytosol, a membrane resulting from the invagination that occurs during the invasion of the erythrocyte by the parasite (the parasitophorus membrane), its own plasma membrane and the vacuole bordered by both these membranes [1]. The capacity of the intraerythrocytic malaria parasite to modify its host cell membrane has already been described in studies that are mainly concerned with protein transport [10,11]. In the case of *Plasmodium falciparum* parasitized human erythrocytes, vesicles, budding off from the parasitophorus membrane have been detected by transmission electron microscopy [33] and by using immunoprobes [34]. A similar vesicle transport system, originating from the parasite and directed towards the host cell membrane, constitutes one of the possibilities to envisage the lipid exchange. Other possibilities to consider are some kind of connection between parasite and host cell membrane, or an intense lipid transfer protein activity.

Such drastic changes in molecular species composition are certainly associated with alterations in particular membrane parameters, such as lipid bilayer fluidity [8,9]. However, and although it is widely accepted that the composition and positioning of the fatty acids attached to the glycerol backbone provides an important mechanism for the regulation of membrane fluidity, it is at present not well possible to ascribe any detailed effects that the above reported changes may have on the physical properties of the host cell plasma membrane. It may be worth mentioning in this context that replacement of the native PC in either intact rabbit or horse erythrocytes for up to 40% by its 16:0/18:2 species, does not affect parameters such as the osmotic fragility, cell morphology, or the in vivo survival of these cells [35]. Replacement of as little as 15% of the native PC by 16:0/16:0-PC, on the other hand, considerably shortens their in vivo survival [35].

As it became clear from this study that *P. knowlesi* infection induces considerable changes in the molecular species composition of both the PC and the PE fractions in the parasitized erythrocyte, the question arose of how these changes are brought about. This question is of particular interest in view of the inability of the parasite, as well as of the host cell, to synthesize fatty acids. It is worth to add that, depending on the stage of parasite development, the total phospholipid content (3100 nmol/10^{10} unparasitized erythrocytes [15]) of the *P. knowlesi* parasitized cell increases by a factor of 2 to 6 [1,36]. This dramatic increase, which essentially comes to the account of the growing parasite, is also accompanied by some changes in the relative amounts of PC and PE, which show an increase from, respectively, 32 and 26% up to 43 and 30% of the total phospholipids [15].

Under normal and healthy conditions, red cell PC is known to be subject to a certain extent of metabolism via two different pathways, i.e., a reacylation of lysoPC that is taken up from the plasma and a direct exchange of intact PC molecules between plasma and erythrocytes [37,38]. Neither of these two routes is likely to provide a major contribution to satisfy the enormous demands of the developing parasite for PC molecules. The first pathway is quite unlikely of being involved

because the parasitized cell is known to possess an active lysophospholipase [39] that will deacylate most of the entering lysoPC. The second route implies either the molecular species composition of the parasitized cells to become very similar to that of the plasma, which is obviously not the case, or – in the case of a preferential uptake of a particular PC species such as 16:0/18:2 – a corresponding decrease in the relative amount of this species from the plasma, which also does not appear to occur (Table II).

The marginal changes that are observed in the molecular species composition of the plasma PC in the infected animals (Table II), similarly cannot account for the alterations that also occur – be it to a more limited extent – in the molecular species composition of the PC in the unparasitized erythrocytes (Tables I and II), and which agree well with recently reported changes in fatty acid composition of these erythrocytes in *P. knowlesi* infected monkeys [40]. These alterations, which mainly concern an increase in 18:2, could be reproduced in vitro, in a *P. falciparum* culture, where they appeared to depend on the presence of a heat labile serum protein [40]. An increase in 18:2 in the unparasitized erythrocytes of infected animals may quite well correspond to the previously observed [41] decrease in the relative amount of 18:2 in the pool of free fatty acids in the plasma of monkeys during *P. knowlesi* infection. A preferential reacylation of 16:0-lysoPC (the most abundant lysoPC species in normal monkey erythrocytes [14]) with 18:2, originating from the plasma, may thus well account for the specific increase in 16:0/18:2 that is observed in the unparasitized erythrocytes (Table II) from infected monkeys. It is of interest to note that the changes induced in these cells appear to be maintained for at least three weeks after starting a treatment with chloroquine, which indicates that the organism allows these cells to be kept in circulation. Whether this alteration in PC molecular species composition disappears when these erythrocytes are sequestered from circulation, remains subject to further studies.

As has already been argued above, it is highly unlikely that this pathway of lysoPC acylation is also responsible for the alteration of the PC molecular species composition in the parasitized cells, the more so as these changes are much greater than in the unparasitized erythrocytes and concern total amounts of lipids that are increased by a factor of up to 6 [1,36]. The two major alternative processes that are known for PC acquisition are the de novo synthesis by the Kennedy pathway, involving the condensation of diglycerides with CDP-choline, and the stepwise methylation of PE [39,42]. When comparing the molecular species composition of the PC in both unparasitized and parasitized erythrocytes (Tables I and II) with that of the PE in the parasitized cells (Table III), it is clear that the *P. knowlesi* induced alterations in PC species cannot result merely from the onset of this PE methylation process, unless the methyl transferases would have some preference for particular species of PE and particularly for 16:0/18:2-PE. On the other hand, there are also a number of observations that emphasize the importance of an active de novo synthesis of PC in *P. knowlesi* parasitized erythrocytes: (i) the enzymes needed for PC biosynthesis according to the Kennedy pathway – choline kinase, cytidyltransferase and cholinephosphotransferase (CPT) – are present in the parasite [39]; (ii) *P. knowlesi* intraerythrocytic development causes an increase in the permeability of the host cell membrane for choline (Ancelin, M.L. and Vial, H.J., unpublished observations); (iii) analogues containing a quaternary ammonium group, that prevents choline entry into parasitized cells, inhibit the novo PC synthesis [43]. Of particular interest to note here, is the observation that the activity of the CPT, the enzyme that catalyzes the last step in PC biosynthesis according to the Kennedy pathway, is strongly dependent on the fatty acid composition of the diacylglycerol involved and decreases in the following order: diacylglycerols prepared from egg PC > 18:0/ 18:1-≅ 16:0/ 18:1-> 16:0/ 16:0-diacylglycerol [44]. As it is known that 16:0/18:1 and 16:0/18:2 are the most abundant species in egg PC, the above diacylglycerol species specificity of the CPT correlates with the alterations that we found in the PC molecular species composition in parasitized cells. In conclusion, these observations seem to suggest that CPT plays an determinant role in establishing the PC molecular species composition that is typical for *P. knowlesi* parasitized monkey erythrocytes.

Regarding the generation of an altered molecular species composition of the PE fraction of the parasitized erythrocyte, it is highly unlikely that this involves the uptake of exogenous PE as this phospholipid accounts for only 1 and 2% of the total plasma phospholipids in healthy and infected monkeys, respectively [32]. The absence of such an exchange process for PE is furthermore illustrated by the fact that, in contrast to what is observed for PC (Table II), the PE in unparasitized erythrocytes from infected monkeys shows a molecular species composition that closely resembles that in the red cells from never infected animals (Table IV). Though a de novo synthesis via the Kennedy pathway has been described to occur also for PE in parasitized monkey erythrocytes [39], its contribution may be marginal because of the virtually complete absence of ethanolamine in the monkey plasma. However, it cannot be ruled out at present that, for this purpose, the parasite produces its own ethanolamine. A third possibility is provided by the decarboxylation of phosphatidylserine (PS), which indeed has been shown to be a major pathway for the production of PE in the parasitized erythrocyte [13,39]. Despite PS evidently appeared to be the most abundantly synthesized phos-

pholipid in the parasitized cell, its steady state level is very low [15], which is probably a consequence of a highly efficient, decarboxylase mediated, conversion of the PS into PE [39,45]. The low amount of PS in the parasitized cell precluded a detailed analysis of its molecular species, though the overall composition of its fatty acids has been determined previously [14]. This fraction appeared to be highly enriched in 20:4, which, assuming a major role for the decarboxylation of PS to generate PE in the parasitized erythrocyte, is in apparent conflict with the marked decrease in those PE species that contain this particular fatty acid in these cells (Table III). In this context, it is tempting to speculate whether PS decarboxylase exhibits substrate specificity, or whether arachidonic acid is withdrawn from PE to be used as a precursor of biologically active derivatives. Further studies will be necessary to elucidate this point. Alternatively our data might suggest that the majority of the PE in the parasitized cell is synthesized via the above mentioned Kennedy pathway. This suggestion seems to be corroborated by the increase in 16:0/18:2-PE, since ethanolaminephosphotransferase, the enzyme that catalyzes the last step in the Kennedy pathway for the synthesis of PE, has a substrate specificity for diacylglycerols which is very similar to that of the CPT (see above), therefore exhibiting a marked preference for 16:0/18:2-diacylglycerol [44].

Finally, it is worth noting that the 70–90% decreases in the relative amounts of 1-alkyl-2-acyl- and 1-alkenyl-2-acyl-PE, which are observed in the parasitized erythrocytes, indicate that the increase in the amount of PE in those cells mainly concerns the diacyl species. In other words, *P. knowlesi* seems to lack the enzymatic machinery to synthesize alkoxyphosphatidylethanolamines.

For its own benefit, Plasmodium parasites are expected to induce changes in their host cell membrane, e.g., to gain better access to nutrients. In this way, the alterations that we reported above can be envisaged as a modulation of the lipid bilayer physical properties, aiming to increase the (protein-, lipid- and/or lipid/protein interphases-mediated) membrane permeability. At the same time, these changes should not result in a stimulation of the host's defense mechanism. Since this does not happen, at least not at an efficient level, several compensatory mechanisms are likely to be operative in the parasitized erythrocytes. It is conceivable that the changes in PC and PE molecular species now detected in *P. knowlesi* parasitized monkey erythrocytes aim to stabilize the host cell membrane, by preferentially synthesizing species that possess a more adequate fitting in the modified membrane.

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