

Lipid Traffic between High Density Lipoproteins and *Plasmodium falciparum*-infected Red Blood Cells

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Abstract. Several intraerythrocytic growth cycles of *Plasmodium falciparum* could be achieved in vitro using a serum free medium supplemented only with a human high density lipoprotein (HDL) fraction ($d = 1.063\text{--}1.210$). The parasitemia obtained was similar to that in standard culture medium containing human serum. The parasite development was incomplete with the low density lipoprotein (LDL) fraction and did not occur with the VLDL fraction. The lipid traffic from HDL to the infected erythrocytes was demonstrated by pulse labeling experiments using HDL loaded with either fluorescent NBD-phosphatidylcholine (NBD-PC) or radioactive [^3H]palmitoyl-PC. At 37°C, the lipid probes rapidly accumulated in the infected cells. After incubation in HDL medium containing labeled PC, a subsequent incubation in medium with either an excess of native HDL or 20% human serum induced the disappearance of the label from the erythrocyte plasma membrane but not from the intraerythrocytic parasite. Internalization of lipids did not occur at 4°C. The

mechanism involved a unidirectional flux of lipids but no endocytosis. The absence of labeling of *P. falciparum*, with HDL previously [^{125}I]iodinated on their apolipoproteins or with antibodies against the apolipoproteins AI and AII by immunofluorescence and immunoblotting, confirmed that no endocytosis of the HDL was involved. A possible pathway of lipid transport could be a membrane flux since fluorescence videomicroscopy showed numerous organelles labeled with NBD-PC moving between the erythrocyte and the parasitophorous membranes. TLC analysis showed that a partial conversion of the PC to phosphatidylethanolamine was observed in *P. falciparum*-infected red cells after pulse with [^3H]palmitoyl-PC-HDL. The intensity of the lipid traffic was stage dependent with a maximum at the trophozoite and young schizont stages (38^h of the erythrocyte life cycle). We conclude that the HDL fraction appears to be a major lipid source for *Plasmodium* growth.

DURING its intraerythrocytic phase, the human malarial parasite *Plasmodium falciparum* reproduces at a rapid rate, completing its development from the infective merozoite to the mature schizont (16–20 nuclei) in <48 h. After the invasion step, the parasite is isolated from the erythrocyte cytoplasm by a parasitophorous vacuole membrane and undergoes a sequential development through a ring form, trophozoite, schizont, and finally the differentiation of 10–20 merozoites. The new merozoites are released into the bloodstream by erythrocyte bursting.

Throughout the schizogonic phase, the parasite sequential development that takes place, corresponds to considerable nuclear and cytoplasmic transformations, including a five-fold increase of total phospholipid content and a decrease in the cholesterol–phospholipid ratio of the infected cell (Holz, 1977; Sherman, 1979; Vial et al., 1982a). Studies of the fatty acid composition have shown that the ability of *Plasmodium* to perform saturation or desaturation reactions of aliphatic chains, as well as chain lengthening and shortening, is limited (Beach et al., 1977). Furthermore, *Plasmodium*

appears unable to synthesize fatty acids de novo from acetate during the erythrocytic cycle (Rock, 1971 a,b; Holz, 1977). These data suggest that parasite growth requires a source of its essential lipids either from the erythrocyte or from the surrounding plasma. As the erythrocyte lipids do not seem sufficient to enable complete parasite growth, the parasite needs to incorporate exogenous fatty acids as well as phospholipids or lysophospholipids (Cenedella, 1968; Rock, 1971b; Vial et al., 1982a,b). *P. falciparum* can be maintained long term in an in vitro culture with a medium that consists of RPMI 1640 and human serum (Trager and Jensen, 1976). Several studies have attempted to grow *P. falciparum* in serum free media. Continuous in vitro culture of *P. falciparum* was obtained in medium containing unsaturated fatty acids adsorbed on serum albumin but the parasitemia was very low in comparison to medium with human serum (Willett and Canfield, 1984). One cycle of growth was obtained in vitro using a medium supplemented with human lipoprotein fractions and dialysable serum factors (Nivet et al., 1983).

In the present study, we have demonstrated that the high density lipoprotein fraction (HDL)¹ is a major lipid source for *Plasmodium* during its erythrocytic life cycle, as shown by the two life cycles obtained in a serum free medium supplemented with human HDL. In contrast, the LDL fraction gives a limited development (<30%) and the VLDL fraction is not able to support any parasite growth. Using HDL labeled with [³H]palmitoyl-PC or with the fluorescent lipid probe, NBD-phosphatidylcholine (NBD-PC), we have also shown the existence of an unidirectional lipid traffic from the HDL to the intracellular parasite. The nonendocytotic mechanism was demonstrated by using HDL labeled specifically on the apolipoproteins with ¹²⁵I and by immunocytochemistry with antibodies against apolipoproteins AI and AII. By pulse experiments with [³H]palmitoyl-PC-HDL on synchronized cultures, the lipid transport was shown to increase from the 24th h of the erythrocytic cycle and to reach a maximum at the 38th h, corresponding to early schizogony. Lipid analysis after [³H]palmitoyl-PC-HDL uptake experiments with or without an exchange procedure, showed that a partial conversion of the phosphatidylcholine (PC) to phosphatidylethanolamine (PE) occurred in infected red blood cells. In addition to the lipid traffic involved in the interactions between the intracellular parasite and its host cell, these results clarify the differential effects of lipoprotein classes on the lipid requirements of *P. falciparum* and the possible control of the parasite erythrocyte cycle by HDLs.

Material and Methods

Chemicals

NBD-PC was supplied by Avanti Polar Lipids, Inc. (Birmingham, USA). ¹²⁵I (100 mCi/ml), [9,10(*n*)-³H]-palmitic acid, [9,10(*n*)-³H]-oleic acid (40–60 Ci/mmol) and phosphorus-32 (1 mCi/ml) supplied by Amersham International (Amersham, UK).

In Vitro *P. falciparum* Culture and Synchronization

Experiments were performed with the *P. falciparum* FcB 1/Colombia and the F 32/Tanzania strains kindly provided by Dr. H. Heidrich (Max-Planck-Institut für Biochemie, Martinsried, FRG) and by Dr. H. Jepsen (Staten SerumInstitut, Copenhagen, Denmark), respectively. In vitro culture was performed according to Trager and Jensen (1976) as modified by Zolig et al. (1982). The basic culture medium contained RPMI 1640, 25 mM Hepes, pH 7.5, 27.5 mM NaHCO₃, 11 mM glucose, reduced glutathione (600 µg/ml), hypoxanthine (50 µg/ml), gentamicin (40 µg/ml), and was supplemented with 5% (vol/vol) human O⁺ serum. Red blood cells (O⁺) were used at a hematocrit of 4%, and the culture was performed under an atmosphere of 3% CO₂, 6% O₂, 91% N₂, at 37°C, with daily medium changes.

The synchronization of cultures was achieved by two successive treatments with 5% (wt/vol) sorbitol at 30–33-h intervals according to Lambros and Vanderberg (1979) or by using the aphidicolin procedure as described by Inselburg and Banyal (1984). Under our culture conditions, the in vitro life cycles were of 40 h for the F 32/Tanzania strain and 48 h for the FcB 1/Colombia strain.

Radioactive Phosphatidylcholine Preparations

[³H]Palmitoyl-PC, [³H]oleyl-PC, or [³²P]PC were prepared from *P. falciparum* cultures labeled with 500 µCi [³H]palmitic acid, 500 µCi [³H]oleic acid, or 250 µCi phosphorus-32, respectively, during a 30–42-h growth period (Rock, 1971). After incubation, two washes with culture

1. **Abbreviations used in this paper:** HDL, high density lipoproteins; NBD-PC, NBD-phosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

medium without serum (600 g, 5 min) were performed and lipids extracted from infected erythrocyte pellets with a chloroform-methanol mixture according to Folch et al. (1957). Phospholipids were separated with chloroform-methanol-ammonia-water (50/20/0.6/0.8; vol/vol) on silica-gel plates (silica-gel 60 precoated TLC plates, E. Merck, Darmstadt, FRG). After iodine revelation, the PC spots were scraped off and eluted with methanol-chloroform, 9:1 (vol/vol).

Preparation of Lipoprotein Fractions

Lipoprotein fractions (VLDL, chylomicron, LDL, and HDL) were prepared by differential ultracentrifugation from fresh healthy human plasma according to Havel et al. (1955). Plasma was centrifuged at 160,000 g (L8/70.1 Ti rotor; Beckman Instruments, Palo Alto, CA) for 24 h at 4°C. The white layer at the top of the tube containing VLDL and chylomicrons was designated as the VLDL fraction and harvested. The remaining portion was brought to *d* = 1.053 by addition of solid KBr and centrifugation was repeated as above. The LDL fraction recovered at the top was carefully collected and the density of the lower part adjusted to *d* = 1.210 with solid KBr. After centrifugation, the HDL fraction was recovered at the top. The VLDL, LDL, and HDL fractions were washed by a new 24-h centrifugation at *d* = 1.006, *d* = 1.053, and *d* = 1.210, respectively. The lipoprotein fractions were dialyzed at 4°C for 2 d against a PBS-EDTA solution (0.15 M NaCl, 5 mM sodium phosphate buffer, pH 7.2, 0.01% EDTA wt/vol). The protein content of the different fractions was determined by the method of Lowry et al. (1951) using BSA as standard and analyzed by 15% SDS-PAGE (Laemmli, 1970). Lipoprotein concentrations were expressed in milligrams or micrograms of protein/milliliter. The storage of the lipoprotein fractions was maintained under sterile conditions at 4°C for <3 wk.

Fluorescent and Radioactive Labeling of HDL

HDL were labeled as described by Martin-Nizard et al (1987). Briefly, 200 µg NBD-PC or radioactive PC (30–70.10⁶ dpm) in chloroform were coated on 400-mg glass beads (80:100; Alltech Associates, Inc., Deerfield, IL) by solvent evaporation under a nitrogen stream. The coated beads were incubated with 2 mg of the HDL fraction in PBS-EDTA, pH 7.2, for 3 h with rotative agitation. Labeled HDL was purified by gel filtration on Superose 12 column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with PBS-EDTA, pH 7.2, at a flow rate of 0.3 ml min⁻¹. After labeling, the absence of PC vesicles in the HDL fraction was attested to by the elution volume of the radiolabeled HDL fraction being similar to that of the native volume. To quantify the [³H]-PC in HDL and its subsequent incorporation in *P. falciparum*-infected red blood cells, HDL lipids were extracted with a chloroform-methanol mixture, according to Folch et al., (1957) and separated by TLC. PC spots were scraped off, eluted with methanol-chloroform, 9:1 (vol/vol) and radioactivity counted as previously described. The organic phosphore content in the PC fraction was determined after acidic hydrolysis according to Ames et al. (1960) and the specific activity expressed in disintegrations per minute/micromole PC.

HDL Protein Iodination Procedure

Protein iodination was performed using the iodine monochloride procedure according to McFarlane (1958): 40% of the total radioactivity was associated with the HDL fraction. Free [¹²⁵I] was removed by gel filtration on a Sephadex G-25 column at a flow rate of 1 ml·min⁻¹ and by a subsequent dialysis against PBS-EDTA, pH 7.2. In the HDL fraction, a discrimination between radiolabeled proteins and lipids was achieved by extraction with a 2:1 (vol/vol) chloroform-methanol mixture (Osborne, 1986). Counting was performed on the two phase extracts with an LKB 1275 minigamma counter (Pharmacia Fine Chemicals). The radioactivity of [¹²⁵I]HDL was mainly associated with proteins and <4% was recovered from the lipid fraction.

Uptake of [¹²⁵I]HDL by 3T3 Fibroblasts

To check that the apolipoprotein iodination procedure did not affect the binding capacity of HDL, the endocytosis of [¹²⁵I]HDL was measured in Swiss 3T3 fibroblasts as described by Pittman et al. (1987). Briefly, cells were incubated overnight in RPMI 1640 with 2 g/liter of NaHCO₃ without FCS. The [¹²⁵I]HDL samples were then introduced into the culture flasks for a 4–6 h period at a final concentration of 100 µg/ml. After incubation, the medium was removed and cells washed three times with PBS. The HDL bound to the 3T3 plasma membrane was removed by incubation with PBS

containing 0.25% (wt/vol) trypsin (bovine pancreas type III; Sigma Chemical Co., St. Louis, MO) and 0.1% (wt/vol) EDTA for 5 min, followed by two washes with PBS by centrifugation (600 g, 5 min). Cell pellets were lysed with distilled water and radioactivity was counted with an LKB 1275 minigamma counter. In parallel, control experiments were performed in the presence of endocytosis inhibitors, under the same conditions, using a preincubation with monensin (5 μ M) or NaN₃ (5 mM) (Sigma Chemical Co., St. Louis, MO) 1 h before addition of [¹²⁵I]HDL.

Uptake and Exchange Procedures with Labeled HDL

Uptake and exchange experiments with NBD-PC-HDL were performed on synchronized *P. falciparum* cultures. Controls were uninfected red cells at the same hematocrit. Cells were first washed twice by centrifugation (600 g, 5 min) with the basic medium without serum and then incubated in the basic medium supplemented with 50 or 100 μ g/ml of NBD-PC-HDL for 30 min at 37°C. After incubation, two procedures were applied on similar aliquots. (a) In uptake experiments, cells were washed twice with basic medium by centrifugation in a microfuge (5,000 g, 1 min; Beckman Instruments) to eliminate free-labeled HDL, and the NBD-PC-labeled cells were then mounted on slides in prewarmed PBS to 37°C, and immediately observed by fluorescence videomicroscopy. (b) In exchange experiments, the incubated cells were then incubated with basic medium supplemented with 1–2 mg HDL/ml or 20% human serum (vol/vol) for at least 30 min at 37°C, with two prewashes in the same medium. After incubation, two final washes were performed, and the cells were examined as above.

The same uptake and exchange procedures were applied to measure the radioactivity incorporated during incubations with 50 or 100 μ g/ml of [¹²⁵I]HDL, [³H]palmitoyl-PC-HDL, [³H]oleyl-PC-HDL, or [³²P]PC-HDL, after which cell pellets were lysed with 200 μ l of distilled water and either directly counted for the ¹²⁵I experiments, or after lipid extraction in [³H]PC-HDL or [³²P]PC-HDL experiments (as described in lipid analysis).

Fluorescence Videomicroscopy

A Nikon Optiphot epifluorescence microscope equipped with a 100 W mercury arc was used with excitation and emission wavelengths for NBD-PC selected by 460–485-nm and 520–560-nm band passfilters, respectively. The resulting images were stored on Sony BRS video cassettes (with a Sony "U" Matic VO5850 P recorder using a LHESA 4036 high sensitivity camera). A 5 \times lens was added to the camera. Digital image processing was performed with the Sapphire system (Quantel, Ltd., Berkshire, UK). Processed images were photographed on PAN F films using the Polaroid videoimage printer.

Lipid Analysis

Cellular lipids were extracted by the addition of 900 μ l chloroform-methanol mixture (1:2, vol/vol) to 200 μ l distilled water lysates of cell pellets. Samples and solvents were vigorously homogenized, left 50 min on ice and centrifuged at 1,000 g for 5 min. Phase separation from the supernatant was achieved by successive additions of 340 μ l 1 M NaCl and 300 μ l chloroform under the conditions defined by Bligh and Dyer (1959). Radioactive incorporation was estimated by counting an aliquot of the organic phase in a Spectral 1214 LKB counter (Pharmacia Fine Chemicals). Phospholipids were separated by TLC on silica-gel plates (E. Merck) according to Mikkelsen et al. (1988) and identified by autoradiography with X-OMAT films and Amplify Spray (Amersham International).

Immunofluorescence Assay

Immunofluorescence experiments were performed on *P. falciparum* infected cells using rabbit polyclonal antisera and monoclonal antibodies directed against the HDL apolipoproteins AI or AII (SERLIA, Pasteur Institut, Lille, France).

After a 30-min incubation with 500 μ g/ml HDL, cells were pelleted and thin smears realized, air-dried, and fixed either with acetone (5 min) at –20°C or with the double methanol/acetone procedure (3 min in methanol, 2 min in acetone) at room temperature. Slides rehydrated with PBS were incubated 1 h with polyclonal antisera (1:200) or monoclonal antibodies (10–50 μ g/ml). After three 10-min washes with PBS, slides were incubated 1 h with FITC-conjugated goat anti-rabbit or anti-mouse immunoglobulins (1:200; Nordic Immunological Laboratory, Tilburg, The Netherlands). After washing, slides were mounted in a PBS/glycerol solution (Citifluor Ltd., London, UK) and examined with a Nikon Optiphot epifluorescence micro-

scope with excitation and emission wavelengths of 470 and 530 nm, respectively.

Western Blotting Analysis

After 30 min incubation with 500 μ g/ml of HDL, free parasites were obtained by a 5-min saponin treatment (0.2% wt/vol in PBS) of infected cell pellets and two washes with PBS (5,000 g, 1 min). Parasite pellets were directly dissociated with 2% SDS–2% mercaptoethanol solution and run on a 15% SDS-PAGE (Laemmli, 1970). Separated proteins were electrophoretically transferred onto nitrocellulose sheets according to the method of Towbin et al. (1979). Polyclonal antisera (1:200) and monoclonal antibodies (10 μ g/ml) directed against apolipoprotein AI or AII were used as primary antibodies and bound immunoglobulins were detected with iodinated [¹²⁵I]-goat anti-rabbit or anti-mouse immunoglobulins (5 \times 10⁵ counts·min⁻¹·ml⁻¹). Autoradiography was performed at –80°C with X-OMAT Kodak film.

Results

In Vitro Growth of *P. falciparum* with HDL

Synchronized cultures of *P. falciparum* (2% parasitemia) at the ring stage were grown in the basic medium supplemented with the different human lipoprotein fractions (VLDL, LDL, and HDL). The parasitemia observed after two life-cycles in the basic medium with 0.50 mg/ml HDL was similar to the parasitemia in basic medium containing 5% (vol/vol) human serum (Fig. 1 A). Under our conditions, the optimal HDL concentrations for the parasite growth were between 0.25 and 0.50 mg/ml (Fig. 1 B). At a concentration of 0.10 mg/ml HDL, only 50% reinvasion occurred while concentrations higher than 1 mg/ml were deleterious for parasite maturation: at 2.50 mg/ml HDL, no reinvasion was observed in the following erythrocytic cycle. In experiments with LDL, the best results were obtained with 0.25 mg/ml LDL (Fig. 1 B) but after two erythrocyte cycles, the parasitemia was only 30% of the control culture medium containing 5% human serum (Fig. 1 A). With the VLDL fraction, no parasite development was observed whatever concentration tested (Fig. 1 A). The results were similar with the F 32/Tanzania strain (40-h life cycle) and the FcB 1/Colombia strain (48-h life cycle). At 0.50 mg/ml HDL in basic culture medium, the parasite maturation was complete (Fig. 2 A), however a 4–6-h delay was observed in comparison to the control medium with 5% human serum (Fig. 2 B). At the LDL optimal concentration, the parasitemia decreased and viable parasites exhibited a delay in the time lapse of schizogony (Fig. 2 C). In the basic medium without serum components or with the VLDL fractions, the parasite maturation was blocked at the trophozoite stage (Fig. 2 D). At a concentration of 0.50 mg/ml HDL no significant lysis of infected red blood cells was observed. In contrast, the LDL and particularly the VLDL fractions induced erythrocytic lysis.

The semidefined medium, supplemented only with the HDL fraction, supported a normal in vitro development of *P. falciparum*. As lipoproteins were the only exogenous source of lipids for the parasite, this medium represents an ideal tool to study lipid traffic between HDL and the intraerythrocytic parasite.

Lipid Traffic in *P. falciparum*-infected Cells: A Unidirectional Transfer from HDL to Parasites

Lipid traffic between HDL and *P. falciparum*-infected erythrocytes was visualized by a fluorescent NBD-PC probe ad-

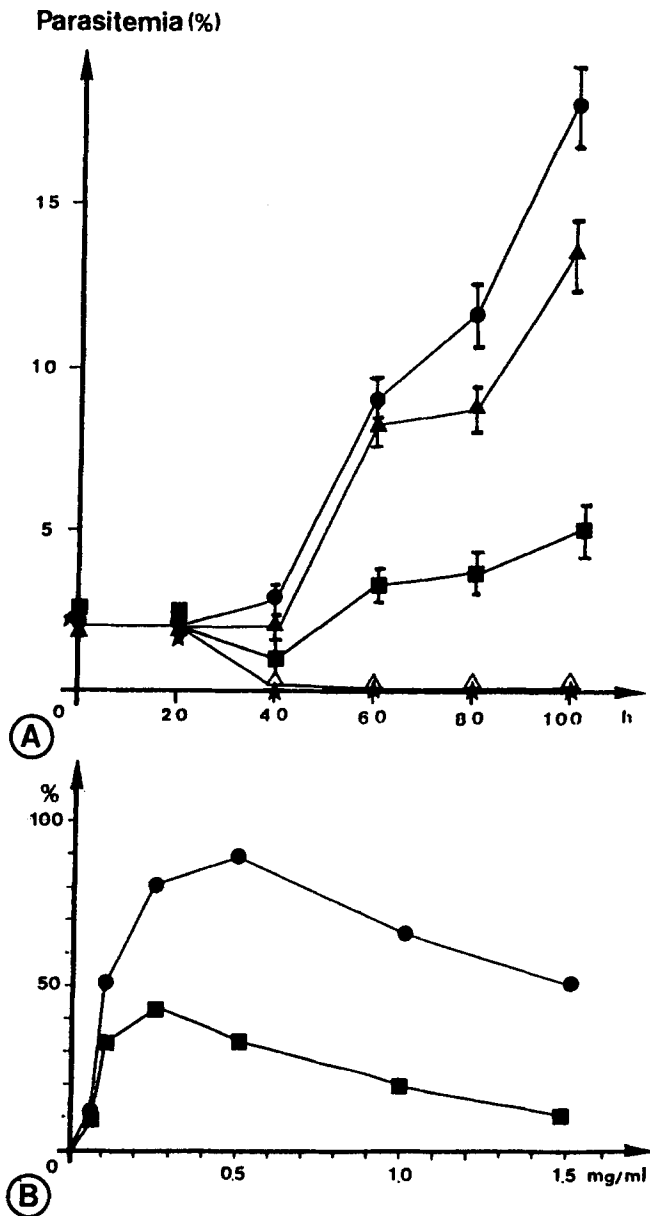


Figure 1. In vitro growth of the F 32/Tanzania *P. falciparum* strain in semidefined medium. The intraerythrocytic life cycle of the F 32/Tanzania *P. falciparum* strain is ~40 h. (A) Comparison of parasitemia under different in vitro culture conditions. Infected red cells (2% parasitemia) at the ring stage were maintained in the basic culture medium without serum (★), or complemented with 0.50 mg/ml of HDL (▲), 0.25 mg/ml of LDL (■), 0.10 mg/ml of VLDL (△), or 5% (vol/vol) human serum (●). Parasitemia was estimated every 20 h on smear slides stained by Giemsa. Medium was changed every day. (B) Influence of the HDL and LDL concentrations on *P. falciparum* development. Infected cells (2% parasitemia) at the ring stage were maintained in basic culture medium supplemented with different HDL (●) or LDL (■) concentrations expressed in mg/ml. Parasitemia was estimated 72 h after HDL addition and expressed as a percent compared with the parasitemia of the control medium containing 5% of human serum. Each point corresponds to the average of three independent experiments.

sorbed to HDL. After a 30-min incubation at 37°C, in the basic medium containing 50 or 100 µg/ml NBD-PC-HDL and two washes in basic medium without serum, the infected erythrocytes displayed a labeling of the intracellular parasites as well as of the erythrocyte membranes (Fig. 3, a-b). The lipid transfer from HDL to parasites was fast since the parasite labeling was observed after only a few minutes. When labeled erythrocytes were washed twice and incubated for 30 min at 37°C in basic medium supplemented with an excess of nonfluorescent HDL (1-2 mg/ml) or 20% human serum (vol/vol), the fluorescence bound to the erythrocyte membranes disappeared both from uninfected and infected cells. In contrast, the fluorescence associated with parasites did not decrease even after 1-h incubation with excess HDL medium (Fig. 3, c-f).

The labeling was associated with the parasitophorous vacuole membrane and/or the parasite membrane as well as with cytoplasmic structures. In infected cells, numerous vesicles (0.2-1 µm diam) and tubular structures were also labeled in the erythrocyte cytoplasm (Fig. 3, a-f). Fluorescence videomicroscopy showed movement of the vesicles between the parasitophorous vacuole and the erythrocyte membrane and a flapping movement of tubular structures.

Lipid Traffic between HDL and P. falciparum Is a Nonendocytic Process

To discriminate between an endocytosis and a nonendocytosis mechanism, experiments were performed using [¹²⁵I]-apolipoproteins-HDL fractions. We previously tested the iodinated HDL endocytosis in Swiss 3T3 fibroblasts as reported by Pittman et al. (1987). HDL was specifically internalized in fibroblasts by an endocytosis mechanism that is inhibited by monensin and NaN₃. Incubations of infected and uninfected erythrocytes with [¹²⁵I] HDL showed saturation kinetics after 10 min (Fig. 4). The complete removal of the radioactivity associated to cells with an excess of either unlabeled HDL (1-2 mg/ml) or 20% (vol/vol) human serum was observed in a few minutes to the same extent in both normal and infected erythrocytes, suggesting a nonendocytosis of HDL apolipoproteins.

This nonendocytosis process of the HDL apolipoproteins was confirmed by the use of rabbit polyclonal antisera and monoclonal antibodies directed against the apolipoprotein A I and the apolipoprotein A II, the major HDL apolipoproteins. By immunofluorescence experiments, no intracellular parasite labeling was observed in infected red blood cells preincubated for 30 min with 0.50 mg/ml of HDL before assay. Furthermore, after electrophoresis and Western blotting no apolipoprotein AI or AII was detected in free parasites isolated by saponin treatment of infected cells incubated with HDL (data not shown). This absence of detection could not be explained by a nonreactivity of the antibodies since similar endocytosis experiments in the 3T3 cells were positive by the immunofluorescence assay.

[³H]Palmitoyl-PC Incorporation in P. falciparum

Similar aliquots of normal and infected red cells were incubated with [³H]palmitoyl-PC-HDL. The kinetics of radioactivity uptake in uninfected erythrocytes were linear during the first 10 min and then reached a saturation plateau (Fig. 5 A). In infected erythrocytes, the first initial rapid

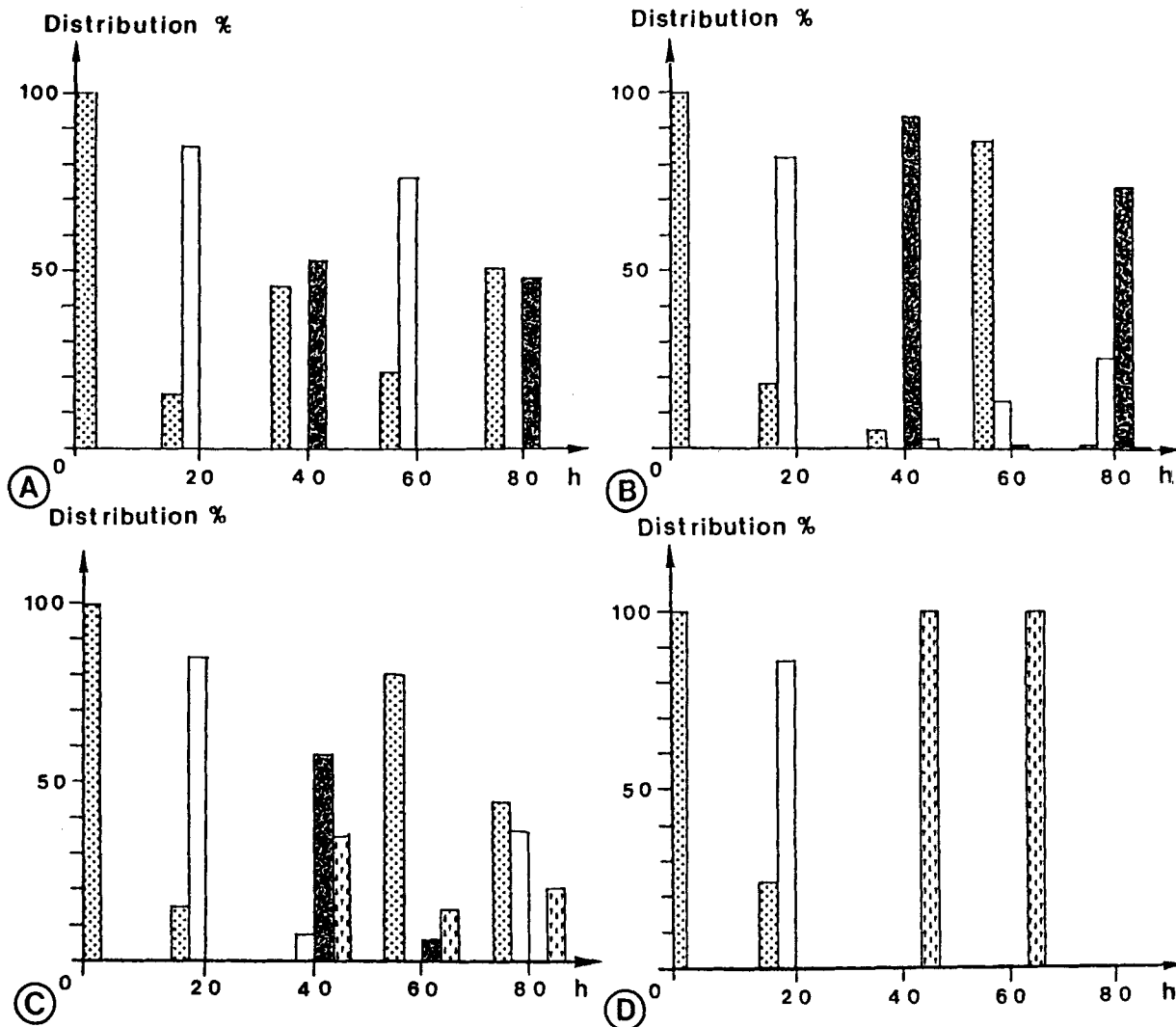


Figure 2. Parasite distribution. In vitro growth of the F 32/Tanzania *P. falciparum* strain in a semidefined medium. The parasite distribution was estimated during two cycles under different in vitro culture conditions. (A) In presence of 0.50 mg/ml of HDL; (B) 5% human serum; (C) 0.25 mg/ml of LDL; (D) 0.10 mg/ml of VLDL. Different stages of parasite distribution: ▨, ring stage; □, trophozoite stage, ■, schizont stage; ▩, dead parasite.

phase was similar to the control. The saturation step, however, was not observed and a slow linear incorporation was maintained for at least 45 min (Fig. 5 A). Exchange procedures with excess HDL (1–2 mg/ml) or 20% serum showed that the radioactivity in uninfected erythrocytes was exchangeable after a few minutes and only 0–5% of the total radioactivity remained bound to the cell fractions (Fig. 5 A). In contrast, in infected erythrocytes ~40–60% of the radioactivity was nonexchangeable (Fig. 5 A). At 4°C, after incubation with [³H]palmitoyl-PC-HDL, similar uptakes between healthy and infected cells were observed with typical saturation plateau and this radioactivity was totally exchangeable (Fig. 5 B).

Lipid analysis after pulse experiments with [³H]palmitoyl-PC-HDL and exchange with excess of unlabeled HDL showed that the radioactivity was transferred from HDL to parasite lipids. By TLC analysis and after the uptake and exchange procedure, the radioactivity was found associated with the PC and PE fractions (Fig. 6 A, lane D). In healthy erythrocytes after incubation with [³H]palmitoyl-PC-HDL, no ra-

dioactivity was found in the PE fraction. To estimate the kinetics of PC/PE conversion, uptake experiments without the exchange step were conducted during a 45-min incubation of schizont-infected erythrocytes with [³H]palmitoyl-PC-HDL or [³H]oleyl-PC-HDL. The lipid analysis showed an early conversion of PC to PE within the first 5-min incubation, but the radiolabeled PE did not exceed 25% of total incorporated radioactivity after 45 min of incubation (Table I). Under our experimental conditions, no intermediate product was observed and similar uptake experiments with [³²P]PC-HDL showed only a radioactive PC fraction (Fig. 6 B).

Lipid Traffic in *P. falciparum*-infected Red Blood Cells Is Stage Dependent

As a disparity of parasite fluorescence intensity was observed in asynchronous cultures with a maximal labeling with late trophozoites and early schizonts, we investigated if there was a difference in the lipid flux according to the developmental stage of the parasite. Synchronized in vitro cul-

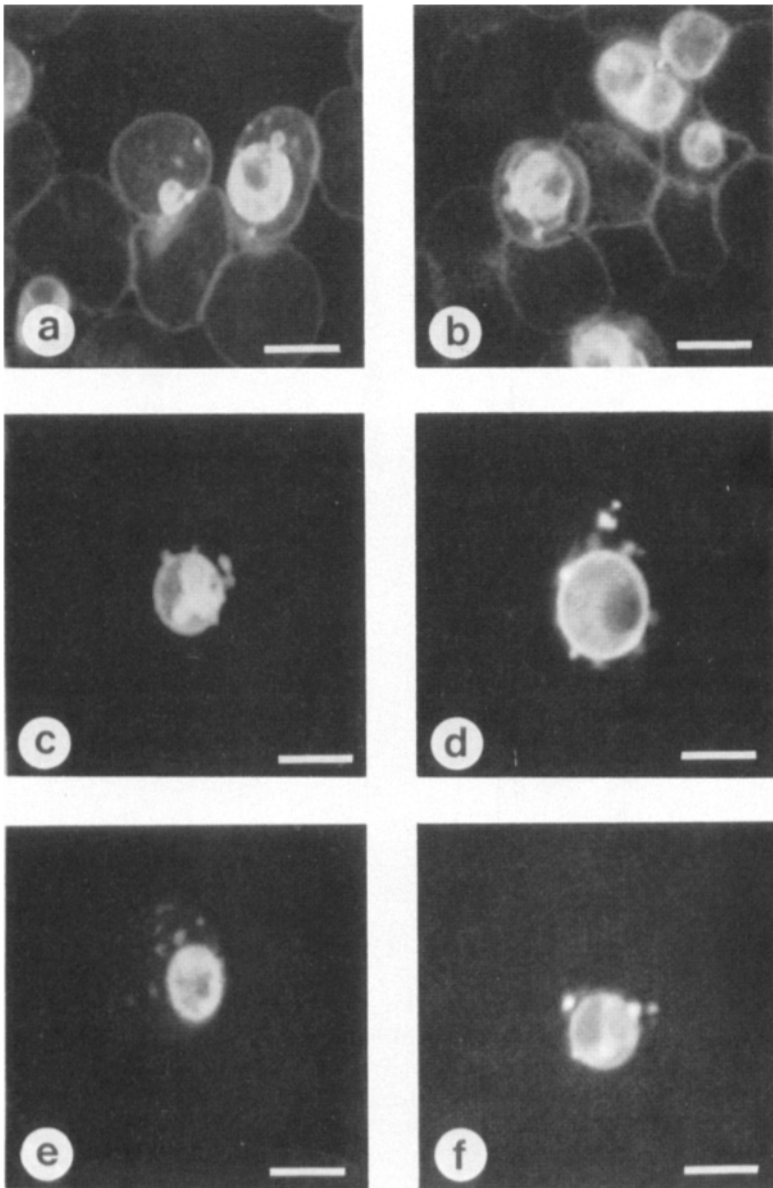


Figure 3. Visualization of the NBD-PC labeling in *P. falciparum*-infected red blood cells during pulse and exchange experiments. Infected cells (10–15% parasitemia) at the trophozoite stage were incubated in 50 or 100 $\mu\text{g/ml}$ NBD-PC-HDL at 37°C for 30 min. Fluorescence videomicroscopy observations were performed after two washes with the basic culture medium without serum (a–b) or after 30 min of incubation with an excess of HDL (1–2 mg/ml) at 37°C (c–f). To avoid the photobleaching of the NBD-PC, all the pictures provided from fluorescent images were recorded with a high sensitivity camera and processed with a digital image system. Bar, 5 μm .

tures were submitted to 30 min pulse labeling with 50 $\mu\text{g/ml}$ of [^3H]palmitoyl-PC-HDL and to 30-min exchange experiments, every 4 h, throughout the 48-h erythrocytic life cycle of the FcB 1 strain (Fig. 7). The lipid traffic determined by the nonexchangeable radioactivity increased after the 24th h and reached a maximum at the 38th h, corresponding to the transition between trophozoite and schizont stages. During late schizogony, the nonexchangeable radioactivity decreased to a level similar to that observed in the ring stage.

Lipid distribution was observed in the parasite after labeling during the optimal lipid traffic period. In vitro *P. falciparum* cultures were synchronized at 4 h, and at the 35th h of erythrocytic cycle cells were incubated with 50–500 $\mu\text{g/ml}$ NBD-PC-HDL for 30 min at 37°C. After the pulse, cells were put back in normal medium containing 5% serum. 1 h later, the fluorescence was mainly associated with the parasitophorous and the parasite membranes (Fig. 8 A). A

few hours later, the fluorescence was distributed around budding or free merozoites (Fig. 8, b–e). The reinvasion process of these labeled parasites was unaffected by incubation with 50–500 $\mu\text{g/ml}$ HDL and was similar to control pulse experiments with the same concentrations of the native HDL fraction or 5% human serum. The fluorescence was not uniformly distributed in the free merozoites (Fig. 8, f). After increasing the contrast by the image processing system, the released merozoites exhibited an asymmetric labeling, most fluorescent lipids being concentrated at one of the merozoite poles.

Discussion

Intraerythrocytic *P. falciparum* development involves the differentiation of 10–20 merozoites in each infected red blood cell every 48 h. As infected erythrocytes are unable

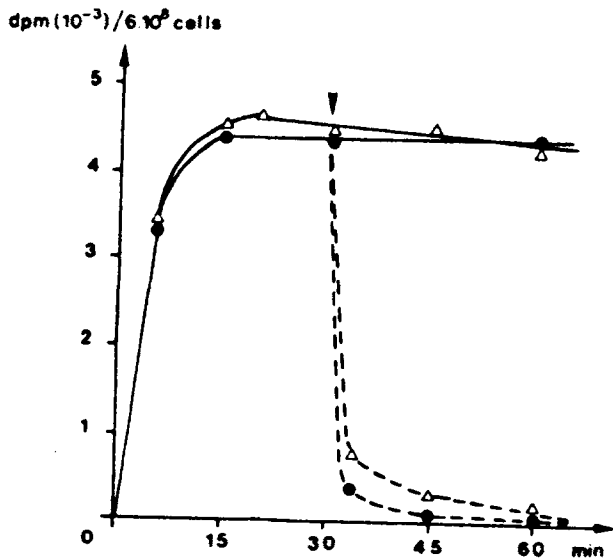


Figure 4. Nonendocytic mechanism of lipid traffic between HDL and *P. falciparum*. Incubation in medium containing HDL with [¹²⁵I]apolipoproteins. Healthy (●) and infected cultures (Δ) at the trophozoite-schizont stage (18% parasitemia) were incubated at 37°C, with 50 μg/ml of [¹²⁵I]HDL (20,000 cpm/μg of protein). The binding kinetics were measured on culture aliquots after two washes with basic culture medium without serum (—). After 30 min of incubation (arrowhead), similar aliquots of infected and healthy cultures were incubated in an excess of native HDL (1–2 mg/ml) or 20% (vol/vol) human serum after first two washes in this medium (— —). Radioactivity associated with cells was measured after water lysis. Each value corresponds to the average of triplicate experiments.

to synthesize fatty acids de novo for new parasite membranes (Holz, 1977), the parasite multiplication requires exogenous lipid, derived from either the host cell or plasma.

Lipids from plasma are mainly supplied by lipoproteins, and, in the present report, the human HDL fraction (*d* = 1.063–1.210) appeared to be a major source of lipids for the parasite. The HDL fraction was the only human lipoprotein fraction that supported in vitro *P. falciparum* growth for two erythrocytic life cycles with a reinvasion rate similar to the standard culture medium supplemented with 5% human serum. The LDL fraction at the optimal concentration allowed a partial parasite growth and no development was observed with the VLDL fraction. However, these different lipoprotein fractions were able to support to the same extent the first nuclear division (Grellier et al., 1990). But, with the LDL fraction only ~30% of parasites were able to undertake a complete schizogony with a normal reinvasion of erythrocytes by merozoites and, with the VLDL fraction, parasites were blocked after the first nuclear division. The LDL and VLDL inhibitory effects on the parasite schizogony could be due either to their particular lipid composition being inadequate for the parasite's lipid requirement or to lytic effects as observed particularly with the VLDL fraction.

Under our experimental conditions, the optimal HDL concentrations for parasite growth were ~5–10-fold higher than the HDL content in the standard culture medium containing 5–10% of human serum. As lipoproteins are complex macromolecules where various serum components could be ad-

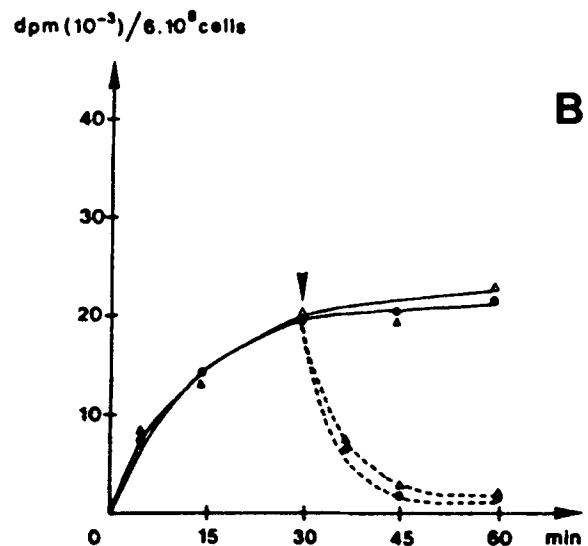
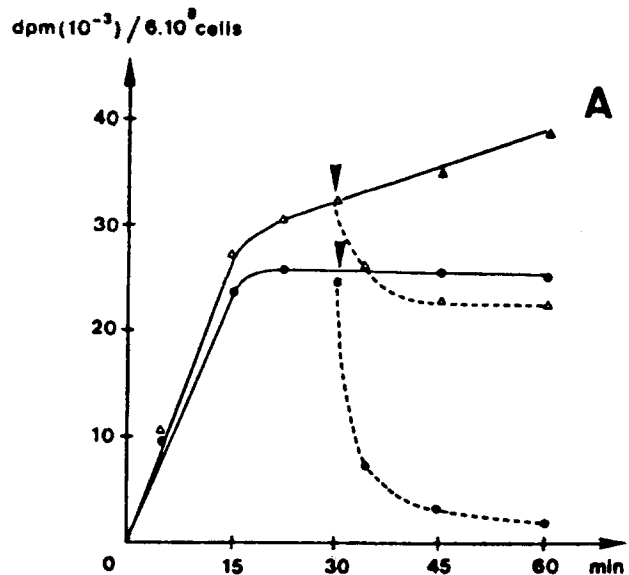


Figure 5. [³H]palmitoyl-PC incorporation from HDL by *P. falciparum* at 37°C (A) and 4°C (B). (A) Kinetics of the PC uptake by the parasites were realized by incubating infected cultures (Δ) at the trophozoite-schizont stage (20% parasitemia) and uninfected cultures (●) at 37°C with 50 μg/ml of [³H]palmitoyl-PC-HDL (9,700 dpm/μg of protein). Each value, from triplicate experiments, was determined after two washes with basic culture medium without serum (—). In parallel experiments, the PC incorporated by the parasites was estimated after 30 min incubation (arrowhead). Aliquots were washed twice and maintained in culture in an excess of HDL (1–2 mg/ml) or 20% (vol/vol) human serum (— —). In each case, cell-associated radioactivity was determined after lipid extraction. (B) PC uptake and incorporation were realized at 4°C as described in A. Infected (Δ) and uninfected (●) cultures; uptake kinetics (—) and incorporated radioactivity (— —).

sorbed and so participate in proliferative effects on eucaryotic cells (Favre et al., 1989; Cuthbert et al., 1989; Jürgens et al., 1989), the high HDL content used in our in vitro culture conditions did not exclude in vivo active roles of other serum components.

Table I. Conversion of PC to PE in *P. falciparum*-infected Erythrocytes

Incubation	5	10	15	30	45
<i>min</i>					
PC incorporated in nmole/6.10 ⁸ cells	1.43	1.60	1.73	2.35	2.83
PC converted to PE in nmole/6.10 ⁸ cells	0.16	0.24	0.35	0.48	0.69
Percent of PC converted to PE	11.5	14.3	20.4	20.7	24.5

Infected red blood cells (25% parasitemia) at the trophozoite-schizont stages were incubated with 100 $\mu\text{g/ml}$ of [³H]-PC-HDL (sp act, 7.05 10⁶ dpm/ μmole PC) at 37°C for 5–45 min. After incubation, cells were washed twice with the basic culture medium without serum, and the lipids were analyzed by TLC and by iodine revelation. Incorporated PC and conversion to PE expressed in nanomoles of PC/6.10⁸ cells were determined after scraping off and elution of the corresponding spots.

The lipid traffic between HDL and the intracellular parasite may be identified by using fluorescent lipids as was done with other animal cells (Pagano and Sleight, 1985). In our experiments, the fluorescent NBD-PC was selected since PC is the most abundant lipid component (74%) of the HDL fraction (Chapman, 1986). Furthermore, NBD-PC does not undergo spontaneous “flip-flop” across the fibroblast lipid bilayer in contrast to phosphatidylserine and phosphatidylethanolamine, and this probe can enter cells by endocytosis (Sleight and Pagano, 1984).

The PC transfer from the plasma HDL fraction to *P. falciparum* did not involve the endocytosis of HDL by the infected erythrocytes. These conclusions were supported by the following data. (a) At 37°C, kinetic studies with

[³H]palmitoyl-PC-HDL showed a biphasic incorporation with an initially rapid uptake in both infected and uninfected cultures and followed by a slow continuous incorporation in infected cultures instead of the saturation plateau observed in normal cells. The first step represents exchanges between HDL and the erythrocyte membrane reaching equilibrium within 15 min, as demonstrated by the saturation plateau observed with healthy erythrocytes. The nonsaturable kinetics measured in infected cells may reflect lipid incorporation by the parasite. Such an accumulation was not observed in infected erythrocytes incubated at 4°C. (b) The HDL apolipoproteins AI and AII were not endocytosed. After an incubation with [¹²⁵I]HDL followed by an exchange procedure with an excess of unlabeled HDL, no radioactivity was recovered from the infected red cells. Furthermore, immunofluorescence and immunoblotting experiments were negative on HDL-incubated cells with antibodies against apolipoproteins AI and AII. (c) The exchange procedure with an excess of native HDL or human serum of HDL pulse-labeled cells indicated that the remaining labeling corresponded to an unidirectional flow of PC from the erythrocyte membrane to the intracellular parasite.

The phospholipid transfer from HDL to parasites was observed throughout the 48-h life-cycle of *P. falciparum* using [³H]palmitoyl-PC-HDL but with different levels of labeling. (a) In the early development cycle, until the 24th h (corresponding to the ring and young trophozoite stage), the PC uptake was constant and weak. (b) From the 24th to 38th h (corresponding to the late trophozoite and early schizont stage), a five- to sixfold increase of incorporation was observed. (c) From the 38th h to the segmented schizont stage, the flow fell to the initial ring stage level. During the

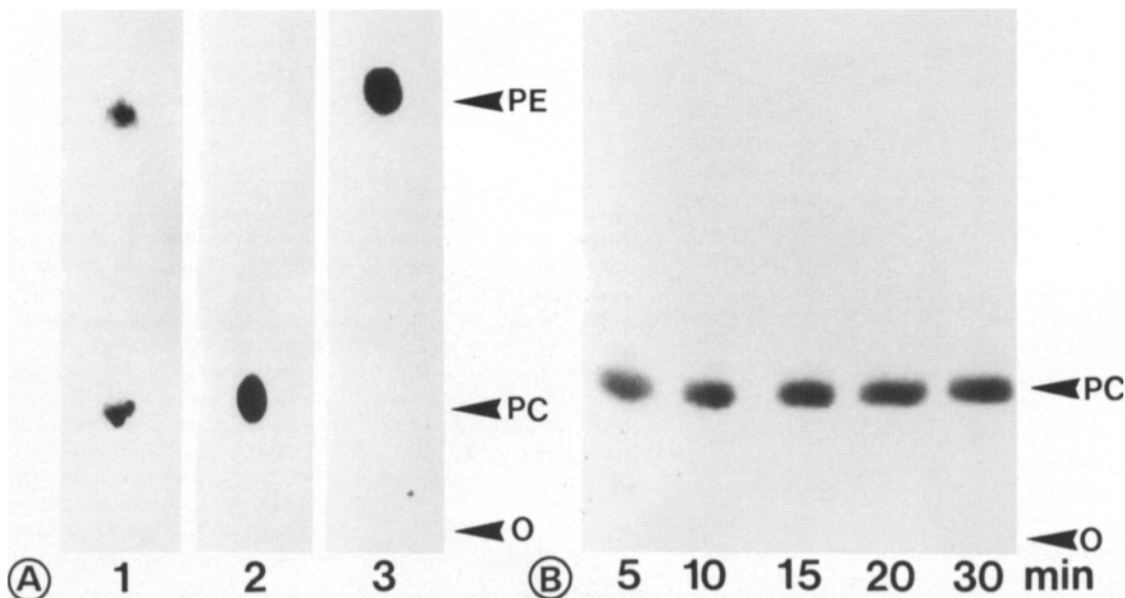


Figure 6. TLC analysis of incorporated lipids after incubation of *P. falciparum*-infected red blood cells with radiolabeled PC-HDL. (A) Lane 1, Conversion of PC to PE after incubation of infected red blood cells (20% parasitemia) at the trophozoite-schizont stage for 30 min at 37°C with 100 $\mu\text{g/ml}$ of [³H]palmitoyl-PC-HDL and after exchange procedure with an excess of HDL (1–2 mg/ml). Lane 2, [³H]palmitoyl-PC control. Lane 3, [³H]palmitoyl-PE control. (B) Lipid analysis of infected red blood cells (25% parasitemia) at the trophozoite-schizont stage after different times of incubation with 100 $\mu\text{g/ml}$ of [³²P]PC-HDL at 37°C and without exchange procedure. (Arrowhead) Origin of migration.

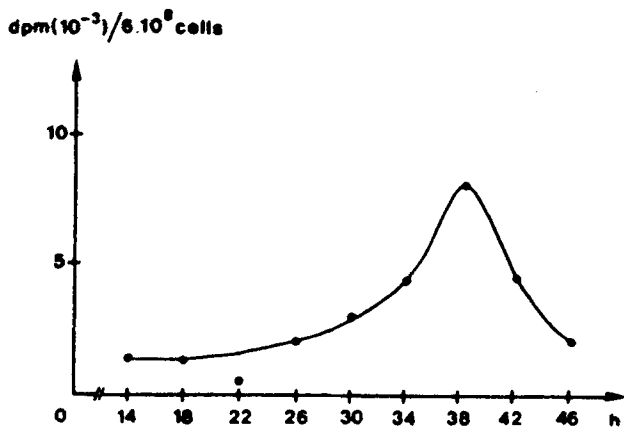


Figure 7. Incorporation of [³H]palmitoyl-PC by the parasite from HDL is stage dependent. Lipid traffic was measured every 4 h during the 48 h of the FcB 1 *P. falciparum* strain life cycle. 4-h synchronized infected cells at the ring stage (15% parasitemia) were maintained in culture with 5% (vol/vol) human serum. The radioactivity incorporated by parasites was determined for infected culture aliquots after two washes with the basic culture medium without serum, 30 min incubation with 100 μg/ml [³H]palmitoyl-PC-HDL (5,200 dpm/μg of protein) at 37°C and exchange procedure at the same temperature in an excess of HDL (1–2 mg/ml) or 20% (vol/vol) human serum. Each point corresponds to the average of triplicate experiments.

24th to 38th h of the life cycle, considerable morphological modifications take place in infected cells. Parasite size increased to fill up almost the whole erythrocyte volume. Numerous nuclear and membranous events take place and structures such as Maurer's clefts are observed in the erythrocyte cytoplasm and are involved in the parasite protein export towards the red cell membrane (for review, see Aikawa, 1988; Hommel and Semoff, 1988). Furthermore, Vial et al. (1982a,b) have shown a considerable increase in phospholipid biosynthesis during trophozoite and schizont maturation. The reduction of PC incorporation from the 38th h could indicate that the parasite accumulates the lipids re-

quired for the biogenesis of its plasma and/or cortical membranes before the final differentiation of merozoites.

TLC analysis of parasite lipids showed a conversion of PC to PE which takes place rapidly, in <5 min, after incubation of infected red blood cells with [³H]PC-HDL. This conversion did not exceed 25% of incorporated radioactivity after a 45-min incubation. Such a conversion differs from previous studies with *P. knowlesi* where this conversion was not observed (Moll et al., 1988). The conversion of PC to PE could be due either to species or to the procedure used to introduce phospholipids into cells. It is known that the procedure can affect the final fate of the phospholipids as demonstrated in Friend erythroleukemia cells (Hohengasser et al., 1986) and in *P. knowlesi* (Moll et al., 1988). In our experiment, PC was adsorbed on HDL and not inserted in vesicles as in *P. knowlesi* experiments (Moll et al., 1988).

Although the PC incorporation in *P. falciparum* did not involve an endocytic mechanism of HDL, this result did not exclude the presence of HDL binding protein(s) on the infected erythrocyte surface. HDL binding proteins have been identified on different cell surfaces and this kind of receptor appears to facilitate cholesterol transport from cells to HDL without endocytosis of HDL particles (Oram et al., 1983, 1987; Tauber et al., 1987). Some specific lipoprotein binding proteins have been described in other parasites: *Trypanosoma brucei* (Coppens et al., 1987, 1988), *T. cruzi* (Prioli et al., 1988), *Schistosoma mansoni* (Rumjanek et al., 1988), and *S. japonicum* (Rogers et al., 1989). Tabas and Tall (1984) have suggested that the lipid exchange between HDL₃ and endothelial cells could also be mediated by hydrophobic interactions between the cell membrane and the HDL₃ surface. Such association may promote lipid transfer. Whatever the mechanism involved in the lipid transfer between HDL and the *Plasmodium*-infected erythrocyte membrane, whether or not it is mediated by a specific parasite protein, the unidirectional flux of PC observed was rapid. The easy reversal of uninfected membrane fluorescence by the exchange procedure could be explained by the fact that the human erythrocyte membrane possesses a protein mediating aminophospholipid translocation, but no PC translocator,

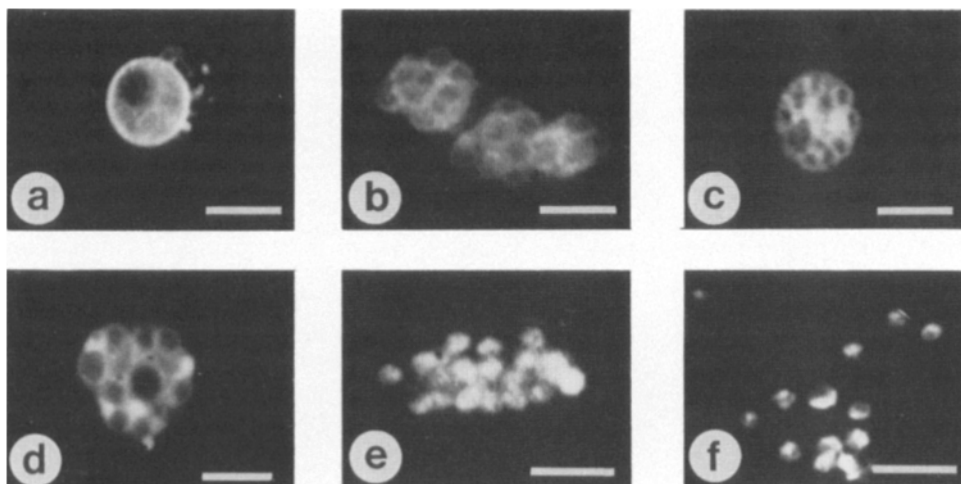


Figure 8. NBD-PC cell labeling during schizogony. Synchronized FcB 1 *P. falciparum* strain-infected erythrocytes at the 35th h of the erythrocytic life cycle were pulse labeled with 100 μg/ml NBD-PC-HDL at 37°C for 30 min and then maintained in a culture medium with 5% (vol/vol) human serum. The fluorescence distribution was observed during the parasite maturation occurring between the 36th h to the 48th h of the erythrocytic life cycle. (a) Young schizont stage; (b, c, and d) schizont stage; (e) segmented schizont with individualized merozoites; (f) fluorescent released merozoites. Bar, 5 μm.

and consequently the PC transbilayer movement is slow (Daleke and Huestis, 1985; Zachowski et al., 1986). In contrast, the rapid PC transport to parasites in *P. falciparum*-infected erythrocytes may implicate a facilitated PC transbilayer movement. This was also observed for the transbilayer mobility of phospholipids in *P. knowlesi*-infected monkey erythrocytes where the half flip-flop time of PC fell from 2–3 h in healthy erythrocytes to <15 min in schizont-infected cells (Beaumelle et al., 1988). Haldar et al. (1989) gave evidence for a protein mediating PC transbilayer flip-flop in *P. falciparum*-infected erythrocyte membranes. The possibility that the lipid traffic between HDL and intracellular parasites involves lysophospholipid derivatives could be taken in account since the lecithin-cholesterol acyltransferase, an enzyme that hydrolyzes the PC in fatty acid and lysolecithin compounds, is associated to the HDL fraction (Chen and Albers, 1982; Cheung et al., 1986) and since it is well known that HDL apolipoproteins (AI, CI, AIV) activate this enzyme (Gotto et al., 1983). The identification of lysoderivatives during the PC to PE conversion could be possible by using radiolabeled PC with higher specific activity than used in these experiments.

Little data on the transport of lipids from the erythrocyte membrane to the parasite is available so far. Haldar et al. (1989) showed that the PC internalization from incubated cells in medium containing PC vesicles was not due to membrane endocytosis but could be achieved by a microdomain pinocytotic activity induced by the intracellular parasite. With a highly sensitive camera coupled with an image processing system used in this study, the videomicroscopy overcomes NBD-PC photobleaching and allows demonstration of numerous fluorescent vesicles and tubular structures in the erythrocyte cytoplasm. The motion of these labeled vesicles between the erythrocyte membrane and the parasitophorous membrane may represent a lipid trafficking pathway induced by the parasite. Tubular structures were always seen to be connected to the parasitophorous vacuolar membrane system and may even reach the erythrocyte membrane, indicating that membrane to membrane contacts might explain the lipid transport. Such a mechanism could be facilitated by lipid transfer protein(s). The existence of vesicles has already been described by electron microscopy for the transport of parasites to the erythrocyte surface via an exocytic process (Howard et al., 1987). However, no parasite lipid exocytosis was observed under our conditions, since red cell membranes were not labeled after incubation with fluorescent HDL, the exchange procedure with native HDL or a further incubation in normal medium (5% serum).

Analysis of the fluorescence associated with free merozoites showed a lipid concentration localized at the apical pole. Lipid concentration was also seen by electron microscopy in the rhoptries of *Plasmodium* merozoites and these lipids may be discharged during the invasion process (Bannister et al., 1986; Stewart et al., 1986). With the fluorescent 12-(9-anthroxyl)-oleic acid, labeling of the merozoite apex was also observed by Mikkelsen et al. (1988), and it has been suggested that the parasite was able to inject its lipids into the host membrane. However, the short time of the reinvasion process and their procedures on fixed cells could not confirm such a dynamic event.

In conclusion, human HDL lipoproteins appeared as a major source of lipids for *P. falciparum* capable of supporting

several erythrocytic life cycles in vitro. Furthermore, the unidirectional flux of lipids from HDL to the intracellular parasite via the erythrocytic plasma membrane has demonstrated a new lipid transport pathway in infected erythrocytes and could open new approaches for antimalarial chemotherapy.

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