NEW APPROACHES IN *IN VITRO* CULTURES OF *PLASMODIUM FALCIPARUM* AND *BABESIA DIVERGENS* BY USING SERUM-FREE MEDIUM BASED ON HUMAN HIGH DENSITY LIPOPROTEINS

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The basic method of Trager & Jensen (1976) for the *in vitro* culture of *Plasmodium falciparum* on human erythrocytes had open a new field in studies of cell and molecular biology of malaria. During its intraerythrocytic phase, the human malarial parasite reproduces in about 48 h the development from the infective merozoite to the mature schizont (segmenters) with 10-20 merozoites. After the invasion step, the parasite is isolated from the erythrocyte cytoplasm by a parasitophorous vacuole membrane. Inside the parasitophorous vacuole, the merozoite undergoes a sequential development through a ring form, trophozoite, schizont with the differentiation of merozoites. These new merozoites are released into the bloodstream by erythrocyte bursting. The intraerythrocytic phase of *P. falciparum* is traditionally maintained *in vitro* on human erythrocytes in RPMI medium supplemented with 10% human serum, under a low O_2 pressure. Several attempts were performed in order to avoid human serum specially in endemic countries, serum contaminations (virus, drugs...), as well as serotype problems. For example, several authors have proposed foetal calf or horse serum instead of the human (Ifediba & Vanderberg, 1980; Divo & Jensen, 1982; Ramos et al., 1986) or substitution of serum by seric fractions: fatty acids adsorbed on bovine serum albumin (BSA) (Willet & Canfied, 1984), human lipoproteins (HLD or LDL) additionned to BSA and human dialysable factors (Nivet et al., 1983). Fairlamb et al. (1985) proposed a serum-free medium composed of RPMI, BSA

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(5 mg/ml), glutamine, methionine, isoleucine, hypoxanthine and horse LDL (68 µg/ml). However, all these attemps did not allowed a parasite growth comparable to that obtained with human serum.

Babesia divergens, the causative agent of bovine babesiosis in Europe, can induce severe infections often fatal in cattle and human (Healy & Ristic, 1988; Gorenflot et al., 1990). We recently described an *in vitro* culture method for *B. divergens* in human erythrocytes that allows high parasitaemia, similar to that observed in splenectomized human patients (Gorenflot et al., 1991). During the *in vitro* erythrocytic life-cyle, *B. divergens* merozoite undergoes the transformation into two or four merozoites in about 8 h (in our culture conditions). In contrast to *Plasmodium sp.*, the development of *B. divergens* takes place free into the erythrocyte cytoplasm (Gorenflot et al., 1991). In few days a parasitemia of about 40% is obtained from a 1% initial parasitemia.

In the course of our studies on neutral proteinases involved in the reinvasion process of red blood cell by *P. falciparum* merozoite, it was important to avoid the seric proteases (especially from the complement cascade) as well as the seric protease inhibitors (a 2 macroglobuline, a 1 antitrypsin...) (Schrevel et al., 1988, 1990). Taking into account the 5-fold increase of total phospholipid content (Vial et al., 1982), the absence of *de novo* fatty acid synthesis in malaria infected red blood cells (Holz, 1977) and the decrease of HDL level in murine or human serum during malaria (Maurois et al., 1980), a strategy was developed in order to determine the effects of the different fractions of human lipoproteins on highly *P. falciparum* synchronized cultures (Grellier et al., 1990, 1991). Furthermore, the

two fold increase of the phospholipid content and the absence of *de novo* fatty acid synthesis in human *B. divergens* infected red blood cells also suggested the necessity of an exogenous lipid source for the parasite growth (Valentin et al., 1991).

HIGH DENSITY LIPOPROTEINS SUPPORT *P. FALCIPARUM* AND *B. DIVERGENS IN VITRO* GROWTH

The human lipoprotein fractions (VLDL, LDL and HDL), prepared from fresh healthy plasma by differential ultracentrifugation (Havel et al., 1955), were tested on *P. falciparum* synchronized cultures at the ring stage. The basic RPMI medium was supplemented with the different human lipoprotein fractions (VLDL: d = 1.006, LDL: d = 1.030-1.053 and HDL: d = 1.063-1.210). Two life-cycles were obtained with 0.50 mg proteins/ml HDL, the parasitemia was similar to the control with 5-10% human serum (Grellier et al., 1991). Under our conditions, the optimal HDL concentrations for the parasite growth were between 0.25 and 0.50 mg/ml. With LDL the best results were obtained with 0.25 mg/ml, but after two erythrocyte cycles the parasitemia was only about 30% to that obtained in the culture medium with human serum. No parasite development was observed with the VLDL fraction, whatever the concentrations tested. Experiments with the F 32/Tanzania strain (40 h life-cycle) and the FcB.1/Colombia strain (48 h life-cycle) as well as with other isolates showed that this serum-free medium can be used on any *P. falciparum* strain so far tested. Addition of LDL to HDL optimal concentration showed a greater stability of *P. falciparum* growth during several erythrocyte life-cycles (unpublished data).

For *B. divergens*, different concentrations of human HDL and LDL were also tested for their abilities to support the parasite *in vitro* growth on human erythrocytes. Here again, HDL alone, at concentrations ranging from 0.25 to 0.75 mg proteins/ml, promoted a significant increase of the parasitemia after 24 h(3 cycles) and 48 h (5-6 cycles) (Valentin et al., 1991). At the optimal HDL concentration, the parasitemia was similar to the culture medium supplemented with 10% human serum during the first 24 h, and about 75% after 48 h in the HDL medium. LDL alone were insufficient for growth enhancement as well as HDL apolipoproteins or HDL lipids adsorbed onto human serum albumin suggesting the HDL integrity for parasite growth *in vitro*.

As lipoproteins were the only exogenous source of lipids for the *P. falciparum* and *B. divergens*, this medium represents an ideal tool for the study of lipid trafficking from HDL to the intraerythrocytic parasites.

LIPID TRAFFICKING FROM HDL TO *P. FALCIPARUM* OR *B. DIVERGENS* INFECTED RED BLOOD CELLS

Lipid trafficking between HDL and parasite infected erythrocytes could be visualized with high sensitive video-microscopy by using a fluorescent NBD-phosphatidylcholine probe (NBD-PC) adsorbed to HDL (Grellier et al., 1991; Valentin et al., 1991). This probe was selected: PC is the most abundant lipid component (74%) of the HDL fraction (Chapman, 1986), and does not undergo spontaneous "flip-flop" across the lipid bilayer in contrast to phosphatidylserine and phosphatidylethanolamine (Sleight & Pagano, 1984). After a 30 min pulse at 37 °C in the basic RPMI medium containing 100 µg/ml NBD-PC-HDL and two washes in basic RPMI, the infected erythrocytes displayed a labeling both of intracellular parasites and erythrocyte membranes. The lipid transfer from HDL to parasite was fast since the parasite labeling was observed after only a few minutes. Exchange procedures (30 min at 37 °C) with an excess of nonfluorescent HDL (1-2 mg/ml) or 20% human serum produce the disappearance of the erythrocyte membrane labeling both from uninfected and infected cells. In contrast, the fluorescence associated with the intracellular parasites did not significantly decrease indicating an unidirectional flux of lipid from HDL to parasites (Grellier et al., 1991). The lipid trafficking between HDL and parasite is a temperature dependent process. Lipid transfer was not observed at 4 °C both with *P. falciparum* and *B. divergens* infected red blood cells.

This lipid trafficking between HDL and intraerythrocytic *P. falciparum* is a nonendocytic process (Grellier et al., 1991). Discrimination between an endocytosis and a nonendocytosis mechanism was performed using HDL specifically radiolabeled with ^{125}I on apolipoproteins and immunochemistry. The incubation of infected and uninfected erythrocytes with (^{125}I)-HDL showed saturation kinetics after 10 min incubation. An exchange procedure with an excess of either unlabeled HDL or 20% serum showed a complete removal of radioactivity associated to cells in a few minutes. The same removal was observed in both normal and

infected cells, indicating a nonendocytosis of HDL apolipoproteins. The nonendocytic process was confirmed by immunofluorescence assays using polyclonal and monoclonal antibodies directed against the HDL apolipoproteins AI and AII. No intracellular parasite labeling was observed. Futhermore, after electrophoresis and Western blotting, AI or AII apolipoproteins were not detected in free parasites isolated by saponin treatment from infected cells incubated with HDL (Grellier et al., 1991).

In *P. falciparum* infected cells, the labeling was associated with the parasitophorous vacuole membrane and/or the parasite membrane as well as with cytoplasmic structures. In infected red blood cells, numerous vesicles (0.2-1.0 µm) and tubular structures were also labeled in the erythrocyte cytoplasm. Fluorescence video-microscopy showed movements of these vesicles and tubular structures between the parasitophorous vacuole and the erythrocyte membrane. These structures could be involved in the lipid transfer from the HDL to the intracellular parasite via the erythrocyte membrane. Recently, Pouvelle et al. (1991) showed that intraerythrocytic *P. falciparum* parasites can endocytose dextrans, protein A and IgG2a antibody. These macromolecules did not cross the erythrocyte or parasitophorous vacuolar membranes, but gain direct access to the aqueous space surrounding the parasite through a parasitophorous duct. Such a situation indicated that these proteins did not use the whole cell surface of infected red blood cells but only ducts. In *B. divergens* infected cells, such vesicles or ducts are not observed. The absence of parasitophorous vacuole in *B. divergens* and the whole labeling of the infected red blood cell membrane indicate that the lipid traffic from HDL to *B. divergens* seems not to be assumed by specific ducts as described by Pouvelle et al. (1991) in *P. falciparum*.

Kinetics of [3H]-oleyl or [3H]-palmitoyl-PC-HDL uptake in uninfected and *P. falciparum* or *B. divergens* infected red blood cells demonstrated a non exchangeable pool of radiolabeled lipid in infected cells. In the first ten minutes, the kinetics of radioactivity uptake were linear and similar both in normal and infected cells. After 15 min, an increasing linear uptake was observed in infected cells indicating the continuity of a lipid uptake in contrast to the plateau with normal erythrocytes. Furthermore, exchange procedures using excess of HDL (1-2 mg/ml) or of serum (20%) dem-

onstrated that 30-60% of the radioactivity incorporated by the infected cells was non exchangeable and only 5-10% in normal cell. TLC analysis of lipids after uptake and exchange showed that the radioactivity was mainly associated to PC. However, a second spot was identified as PE both in *P. falciparum* (Grellier et al., 1991) and *B. divergens* (Valentin et al., 1991). This PC/PE conversion did not exceed 25% in *P. falciparum* infected cells. The activation of a LCAT (Lecithin-Cholesterol-Acyl-Transferase) enzyme present in the HDL fraction (Cheung et al., 1986) could explain such a conversion. However, Phospholipase-A activity, followed by reacylation or transacylation could also lead to this conversion (Kramer et al., 1984).

In *P. falciparum* infected cells, the lipid uptake is stage-dependent (Grellier et al., 1991). In the early erythrocytic cycle, until the first 24 h corresponding to the ring and young trophozoites stage, the PC uptake was weak and constant. From the 24th to the 38th hour, corresponding to the late trophozoite and early schizont stage, a five-to six-fold increase of radioactivity incorporation was observed. From the 38th hour to the segment schizont stage, the lipid flow fell to the initial ring stage level. These results are consistent with the considerable morphological modifications which take place between the 24th to 38th hour of the erythrocytic cycle. During this period, parasite size increases 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 (Aikawa, 1988; Hommel & Semoff, 1988). During trophozoite and schizont maturation, the increase of lipid trafficking in *P. falciparum* infected red blood cells appears parallel to the considerable increase in phospholipid biosynthesis (Vial et al., 1982). The reduction of lipid incorporation after the 38th hour could indicate that the biogenesis of membranes is engaged for the merozoite differentiation.

IN VITRO INDUCTION OF *P. FALCIPARUM* SCHIZOGONY BY THE HUMAN HDL

The successful development of *P. falciparum* in a serum-free medium based on human HDL arise the question of the regulatory signals which could control the different steps of *P. falciparum* erythrocytic cycle: transformation from ring to trophozoite stage, induc-

tion of schizogonic nuclear divisions, cell morphogenesis and release of merozoites. To estimate the effects of lipoprotein fractions on the *P. falciparum* development, we have developed a viability test combining two fluorescent probes: bisbenzimide H33258, a specific DNA probe (Howard et al., 1979) and rhodamine 123, a fluorescent marker known to accumulate in proton compartments, especially in the mitochondria of living cells (Divo et al., 1985).

Using highly synchronized culture of segmented *P. falciparum* schizonts, it was shown that, in the absence of serum, merozoites were released from schizonts and the erythrocyte invasion was normal. Furthermore, the parasites were able to differentiate in ring and young trophozoite but unable to undertake schizogony (Grellier et al., 1990). Addition of 5% serum at different periods of the first 24 h *P. falciparum* development in RPMI medium showed that the trophozoite maturation in schizont was restaured and a erythrocyte reinvasion rate similar to controls. After 24 h of culture, the parasitemia fell in agreement with the decrease of viability. After 32 h of culture without serum, addition of serum did not allow the re-establishment of schizogony (Grellier et al., 1990).

Addition to young trophozoites developed in absence of serum during 30 h of the different lipoprotein fractions HDL, LDL, VLDL), at the optimal concentrations defined previously, stimulated the early schizogony giving the first nuclear division. However, only the HDL fraction was able to assume a complete schizogony and erythrocyte reinvasion. With LDL, about 30% of parasites were able to perform a normal schizogony and reinvasion. With VLDL, all parasites were blocked after the first nuclear division. Similar results were obtained with the *P. falciparum* FcB.1/Colombia and F32/Tanzania strains which exhibit cycles of 48 and 40 h respectively. No nuclear division was observed when HDL or LDL apolipoproteins were added to the culture medium.

The reinvasion process and the first 24 hr of the erythrocytic cycle (differentiation of merozoite in ring and young trophozoite) appear independent of serum components. This period is characterized by a weak metabolic activity (Scheibel & Sherman, 1988) and could correspond to the G_0 phase of eukaryote cellcycle (Ginsburg, 1990). HDL, LDL and VLDL allowed the first nuclear division and such re-

sults are in agreement with the proliferation induced by lipoproteins (Cuthbert & Lipsky, 1989). In addition to their contribution for membrane biogenesis, the lipoproteins could play a role in transduction or proliferative signals as recently shown by the phosphatidylcholine turnover products on cell proliferation (Van Corven et al., 1989).

In conclusion, the discovery of human HDL effects on *P. falciparum* and *B. divergens* erythrocytic development gives the possibility to purify easily exoantigens or parasites proteins. It also open new powerful trends in cell and molecular biology of malaria and babesiosis as well as in chemotherapy strategies. For example, the purification of exoantigens in serum free medium based human HDL avoid upon the interferences of seric proteins which are in high concentrations (serum albumin, immunoglobulines...). The possibility to block the parasite development at the young trophozoite stage and to reinitiate a complete schizogony by serum or HDL addition, gives an experimental model for study the gene expression during the cascade of events in *P. falciparum* schizogony. Low and high density lipoproteins appear efficient carriers of anticancer porphyrins used in the photodynamic therapy (Maziere et al., 1990). The possibility of using photoirradiation sensitized by mero-cyanine 540 to purge *Plasmodium* infected blood of mice was described recently by Smith et al. (1991). New lipophilic photosensitizers or drugs targeted by lipoproteins could help to open new chemotherapeutic strategies.

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