Mitochondrial Heter ogeneity in Human Malarial Parasite *Plasmodium falciparum*

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Received 11 Feb 1999

ABSTRACT Mitochondria of the human malarial parasite *Plasmodium falciparum* in sexual blood stages (or gametocytes) had been structurally different from those of asexual blood stages of their life cycle in human host. We report here the existence of mitochondrial heterogeneity based on their characteristics of ultrastructural morphology in the asexual and sexual blood stages of *P. falciparum* from *in vitro* continuous cultures. Mitochondria in the sexual stage-parasites were more numerous and contained a greater density of cristae than the organelles in the asexual stage-parasites. It was demonstrated that there were apparent variations in size and appearance of the mitochondria between the male and female parasites of the sexual gametocytic stages. Mitochondrial oxygen consumption of the sexual stage-parasites was relatively low, and it was not different from the asexual blood stage-parasites. However, both stages of the parasites' growth and their oxygen consumption were found to be sensitive to atovaquone, cyanide and 5-fluoroorotate which were inhibitors of mitochondrial electron transport system and pyrimidine biosynthetic pathway, respectively. Therefore, the role of mitochondrial organelles with different morphological properties in the asexual and sexual stages of parasite's development remains to be elucidated.

KEYWORDS: malaria, *Plasmodium falciparum*, gametocyte, mitochondria.

INTRODUCTION

During the asexual blood stage the human malarial parasite, *Plasmodium falciparum*, grows and matures within the erythrocyte of the human host. The absence of cristate structure in mitochondrial organelle at this parasite stage has been demonstrated from electron microscopic studies.[1-5] Energy requirement is provided by metabolizing glucose primarily by anaerobic glycolysis.[6,7] It has been clearly evident from biochemical and enzymatic studies that the asexual stage parasite has mitochondrial electron transport system and oxygen-requiring system that is necessary for parasite's growth and multiplication.[3-10] In addition to the development of the asexual blood stages, *P. falciparum* contains sexual blood stages in the human host necessary for survival and transmission into the mosquito vector to complete its life cycle. Relatively little is known concerning cristate structure and biochemical function of the mitochondrial organelle in the sexual blood stage of parasite development,[2,11,12] although it develops within the erythrocytes of human host.

In this communication, the ultrastructural characteristics of mitochondrial organelles based on the electron microscopic studies of asexual and sexual blood stages of *P. falciparum* grown *in vitro* were investigated. The biochemical properties of the sexual stage parasites were compared to the asexual blood stage parasites.

MATERIALS AND METHODS

Malarial parasites

Human malarial parasite *P.falciparum* (T_9, NP_{10} and KT_3 isolates) was cultivated from the frozen sample in sorbitol-glycerol cryoprotectant[13] by the candle jar method of Trager and Jensen[14], using 5% hematocrit of human erythrocytes group 'O' in the RPMI 1640 medium supplemented with 25 mM N-2-hydroxylethylpiperazine-N'-2-ethanesulfonic acid (Hepes), 32 mM $NaHCO_3$ and 10% human serum group 'O'. Cultures started at low parasitemia (~1-2%) were changed with medium twice daily until the cultures had ~30% parasitemia and then parasites were harvested. Synchrony of the culture to get mainly trophozoite stages was performed by the sorbitol procedure of Lambros and Vanderberg.[15] For the sexual blood (gametocytic) stages, either NP_{10} or KT_3 isolate was used as gametocyte-producing strain and then induced according to Ifediba and Vanderberg.[16] Approximately 4% parasitemia of mixed stages gametocytes were routinely obtained

on day 10-15 of cultivation after adding normal fresh erythrocytes. The gametocytic stages were purified using Percoll step-wise gradient centrifugation according to Knight and Sinden.[17] Parasites were freed of their host erythrocytes by incubating in an equal volume of 0.05% (for sexual gametocytic stages) or 0.15% saponin (for asexual blood stages) in the RPMI 1640 medium at 37°C for 20 min, and washed at least 3 times before experiments.

Microscopic examination of *P. falciparum* morphology

Transmission electron microscopy (TEM) of the infected erythrocytes at the asexual and sexual gametocytic stages was performed according to the method of Sinden.[11] The processed samples were examined with a JEOL-100SX transmission electron microscope at the Center of Scientific and Technological Research Equipment of Chulalongkorn University, Bangkok. Light microscopy (LM) of the infected erythrocytes was examined on methanol fixed and Giemsa's stained parasites by a Nikon labophot-2 microscope.

Measurement of oxygen consumption by *P. falciparum*

The rates of oxygen consumption for a consistent number of host cell-free *P. falciparum* at both stages of parasite development were measured in a modified medium[4] containing 75 mM sucrose, 225 nM mannitol, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 1 mM ethylene glycol bis(β-aminoethyl)-N-N-tetraacetic acid, 5 mM Hepes, pH 7.4, by using a Clark-type oxygen electrode and YSI oxygen monitor according to the method of Robinson and Cooper.[18] The oxygen uptake in a chamber with volume of 3 ml was followed for 3-5 min and recorded at 37°C with a temperature-controlled circulator. Mitochondrial inhibitors at desired concentrations were tested against oxygen consumption of the two stages of the parasites by injecting into the reacting chamber. The rates of oxygen consumption by these parasites were then followed for the next 3-5 min. The 50% effective concentration (EC_{50}) was defined as the concentration of the inhibitor causing 50% inhibition of the parasite oxygen consumption, compared to the control parasite oxygen consumption without inhibitor.

***In vitro* antimalarial test**

Antimalarial activity against asexual stages of *P. falciparum in vitro* (blood schizontocidal activity) was quantified by measuring % parasitemia in a four-day culture in the presence of the tested compounds at various concentrations.[19] All compounds were tested in triplicate at each concentration used. The 50% inhibitory concentration (IC_{50}) was defined as the concentration of the compound causing 50% inhibition of the parasite growth in a 4-day culture, compared with the compound-free control of the parasite culture.

Gametocytocidal activity against *P. falciparum in vitro* was tested by measuring % parasitemia in a 24-well microculture plate in the presence of various concentrations of tested compounds for 2 days starting at day 5 of cultures after provision of fresh erythrocytes. At day 7 and again at day 8, all wells received fresh medium without the compound. Thin blood films were prepared at day 9. This represents a four-day period of gametocyte development in microculture during the antimalarial test, the first two days under pressure of test compound. The test method and IC_{50} value calculation were essentially from Bhasin and Trager.[20]

Results and Discussion

The *in vitro* cultures of T_9, NP_{10} and KT_3 isolates (all multidrug-resistant parasites) of *P. falciparum* were taken from frozen samples in sorbitol-glycerol cryoprotectant, and continuously induced for production of the sexual gametocytic stages for over 2 years. It was noted here that the establishment of new cultures (or subcultures) by dilution with fresh erythrocytes markedly reduced gametocyte production even in the presence of inducing conditions as described by Ifediba and Vanderberg[16], and this might result in loss of gametocytic stages in those cultures. T_9 parasite was found to be no longer a gametocyte-producing strain. This may result from a developmental defect during maturation that has arisen during the long-term cultivation of the asexual stages *in vitro* (~ four-year culture from 1990-1994), as previously described in the different isolates of *P. falciparum* by Guinet *et al.*[21]

NP_{10} and KT_3 parasites had been cultured for more than 2 years and they could be induced for gametocytogenesis to give parasitemia of the sexual stages as high as ~4%. The gametocytes were purified from the asexual stage parasites by the established method of Percoll step-wise gradient centrifugation. The purified gametocytes used for TEM and oxygen consumption are demonstrated in low magnification of Giemsa's stained parasites by LM in Fig 1. Mixed populations of male and female gametocytes of stages III-V existed in the parasites' preparations. The developmental stages of gametocytes were divided into 5 stages according to the cytological classification of Hawking *et al.*[22]

The mitochondrial organelles observed in *P. falciparum*, described as double membranous structure, were by no means unique between the asexual and sexual development stages in that they contain tubular-like cristate structures (Figs 2-5). The mitochondrial organelles in the asexual blood stage parasite (Fig 2) were never as numerous as they were in the sexual gametocyte stage (Figs 4 and 5). Fig. 2 shows a clearly defined double membrane structure of a mitochondrion in the trophozoite stage of the asexual blood parasite. Like other protozoan mitochondria, they are surrounded by a double membrane, and the inner membrane gives rise to the tubular cristae. The elongated organelle showed a limited number of tubular cristae (Fig 3). All mitochondrial organelles, so far examined, in the asexual blood stages had no or little internal membranous whorls rather than well-defined tubular cristae. They are classified as 'type I' mitochondria. In addition to the unique morphological characteristics of 'Type I' mitochondria in the asexual stage, the maturing female gametocytic stage parasite (macrogametocyte) had numerous mitochondrial organelles (more than 5) containing marked proliferation and pronounced infolding of the inner membrane giving rise to large numbers of tubular cristae with clear intracristal space (Fig 4). They are classified as 'Type II' mitochondria (Figs 6-9). All macrogametocytes examined had both 'Type I' and 'Type II' mitochondria (Fig 6). Most mitochondrial organelles in the macrogametocytes were 'type II' mitochondria. Higher magnification of these "Type II' organelles showed them to have different forms in their shapes of the tubular cristae, for instance, membranous whorls cristae (Fig 6), or well separated tubular cristae (Fig 7), or finger-like cristae of closely folded inner membrane (Figs 8 and 9). By comparison with the maturing male gametocytic stage (microgametocyte) (Fig 5), it was noted that the developing male parasite had less numbers of the organelles (less than 5), possessed higher numbers of the cristae, and contained electron-dense tubular cristae (Figs 10 and 11). They are classified as 'Type III' mitochondria.

Based upon these findings, it is concluded that 1) the male gametocytic parasites have numerous 'Type III' mitochondria and their mitochondria are less numerous than those of the female parasites, 2) the female parasites contain mainly populations of 'Type II' mitochondria and a limited number of 'Type I' mitochondria, and 3) the asexual blood parasite has a single mitochondrion associated with 'Type I' organelle (Table 1). Our results reported are consistent with the observation[23] in the other apicomplexan blood parasite *Haemogregarina myoxocephali* at different numbers of mitochondrial organelles in various developmental stages of its life cycle: the erythrocytic stage, ~1-2 organelles; the sexual stage, ~4-6 organelles; and the sporozoites, ~8-10 organelles. The existence of such variations in mitochondria of male and female gametocytic stages, both in terms of their numbers and in the density of cristae, suggests that these developing sexual stages have high demand for energy transduction and also metabolic activity differences from the asexual stages. These active mitochondria in the sexual stages may be necessary for their survival during transmission into the mosquito vector.

Table 1. The comparison of type and number of mitochondrial organelle between asexual and sexual blood stages of *P. falciparum*.

Stages of parasite	Mitochondrial characteristics	
	Type	Number
Asexual stage	I	1
Sexual stage		
Female	II >>I	>5
Male	III	<5

To see whether the mitochondria in sexual gametocytic stages of *P. falciparum* were biochemically active or not, mitochondrial oxygen consumption of the parasites were performed with known mitochondrial inhibitors. It was found that mitochondrial oxygen consumption of cultured *P. falciparum* from both stages were not different (Table 2). They had relatively low activity (~ 200 - 240 nmol/min/10^8 parasites), compared to the human leukocytes which had an oxygen consumption of 1,090 nmol/min/10^8 cells (n=2). Cyanide, a known inhibitor of mammalian electron transporting complex IV (cytochrome c oxidase),[24] had an inhibitory effect against oxygen consumption of the host cell-free parasites isolated from both asexual and sexual stages (Table 2). At a concentration of 1.0x 10^{-3} M, cyanide inhibited 52% and 88% of the oxygen consumption by the asexual and sexual stage parasites, respectively. Cyanide has been shown to exhibit low antimalarial effect against *P. falciparum* growth *in vitro* with IC_{50} value in the micromolar level.[8,25] It has inhibitory effects against the purified cytochrome c oxidase of both *P. berghei*[4] and *P. falciparum*.[10]

The parasite oxygen consumption of both stages was found to be sensitive to atovaquone inhibition

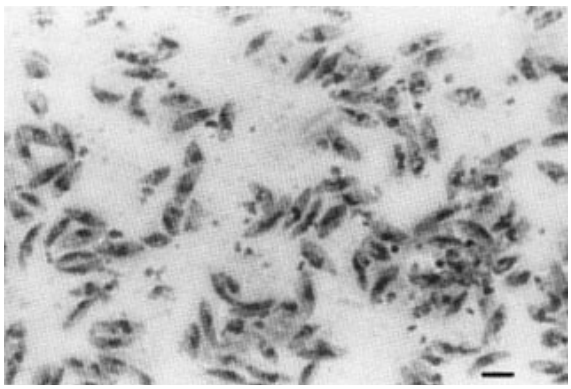

Fig 1 Light micrograph of purified gametocytes from *in vitro* cultures of *P. falciparum*. All preparations were methanol fixed and stained with Giemsa's stain. The bar represents 10 µM.

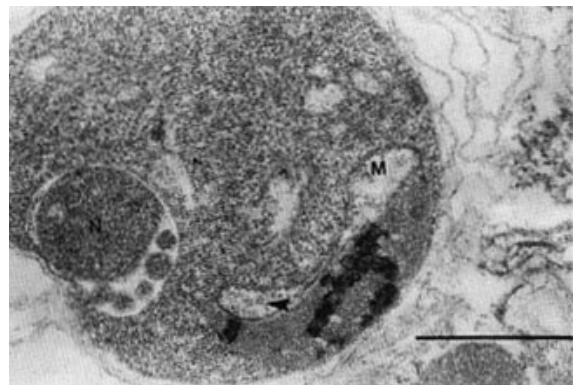

Fig 2 Transmission electron micrograph (TEM) of the asexual trophozoite stage of *P.falciparum*. The mitochondrion shows a clearly defined double membranes and an elogated form which is prepared for binary fission. A membrane whorls-like tubular crista resulting from an underdeveloped inner membrane is marked by an arrowhead. The bar represents 1 µM. N, nucleus; M, mitochondria; P, crystalline pigment.

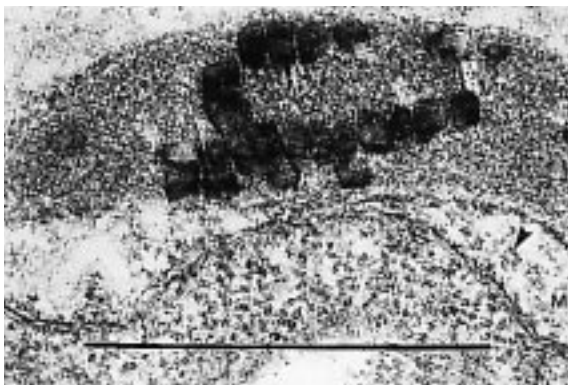

Fig 3 TEM of higher magnification of 'Type I' mitochondria in the asexual trophozoite stage of *P. falciparum*. Only one tubular cristate structure is demonstrated (arrowhead). The bar represents 1 µM. M, mitochondria; P, crystalline pigment.

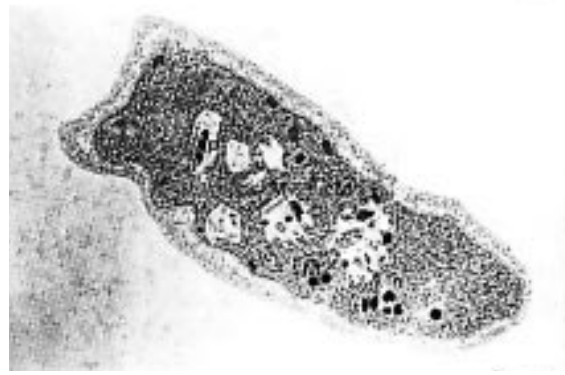

Fig 4 TEM of maturing female gametocytic stage (macrogametocyte in stage IV) of *P. falciparum*. Numerous 'Type II' mitochondria are observed. The bar represents 1 µM. M, mitochondria; P, crystalline pigment.

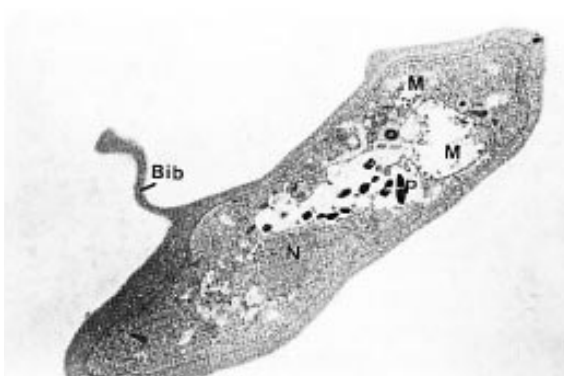

Fig 5 TEM of maturing male gametocytic stage (microgametocyte in stage IV) of *P. falciparum*. Few 'Type III' mitochondria are observed, Laveran's 'Bib' is also visible at the middle left. The bar represents 1µM. N, nucleus; M, mitochondria; P, crystalline pigment.

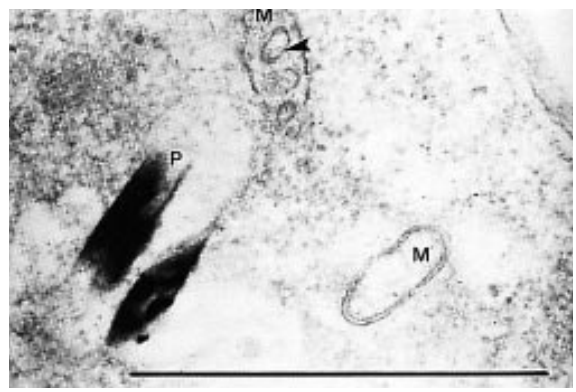

Fig 6 A high magnification TEM of mitochondria in a maturing female gametocyte. The lower (Type I) and the upper (Type II) mitochondria are shown. The tubular cristae of 'Type II' organelle is marked by an arrowhead. The bar represents 1 µM. M, mitochondria; P, crystalline pigment.

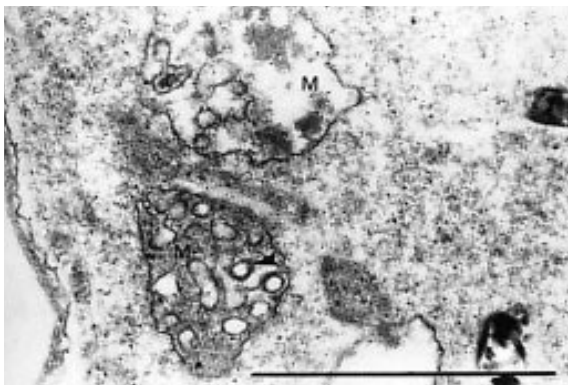

Fig 7 TEM of 'Type II' mitochondria in a female gametocyte. Two organelles are observed with high magnification. They contain numerous tubular cristae, marked by arrowhead. The bar represents 1 µM. M, mitochondria.

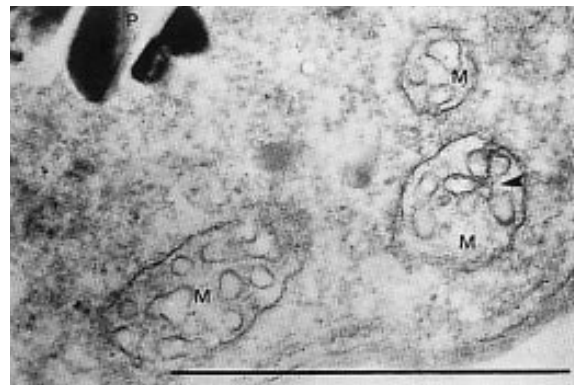

Fig 8 TEM of variant forms of 'Type II' mitochondria in a female gametocyte. Three organelles are observed with high magification. Numerous tubular cristae resulting from the extensively folding of the inner membrane are observed. An arrowhead indicates the finger-like cristae. The bar represents 1 µM. M, mitochondria; P, crystalline pigment.

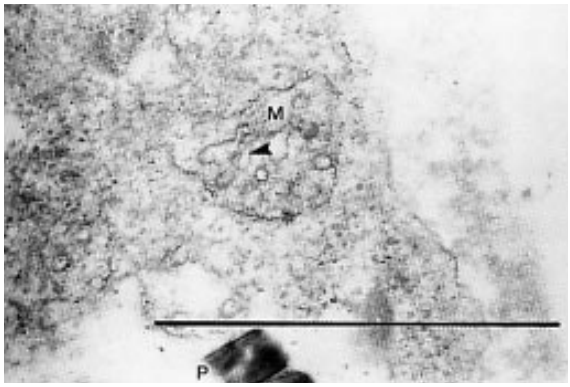

Fig 9 TEM of another variant forms of 'Type II' mitochondria in a female gametocyte. The finger-like cristae is typically found in the female gametocytes (arrowhead). The bar represents 1 µM. M, mitochondria; P, crystalline pigment.

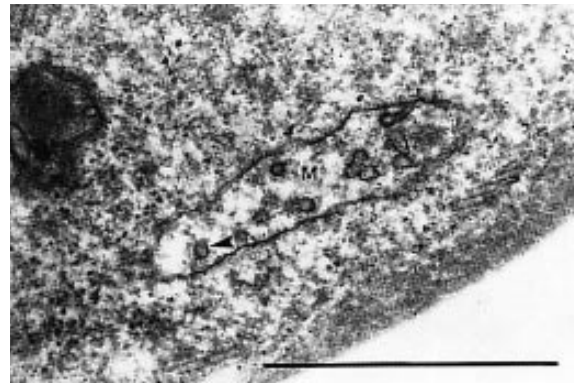

Fig 10 TEM of 'Type III'mitochondria in a male gametocyte. Electron-dense and finely compact of the tubular cristae is typically associated with this type of the organelle. The numbers of cristae in 'type III' mitochondria are more than those of "Type II' mitochondria of the female gametocytes. The bar represents 1 µM. An arrowhead indicates electron-dense tubular cristae. M, mitochondria.

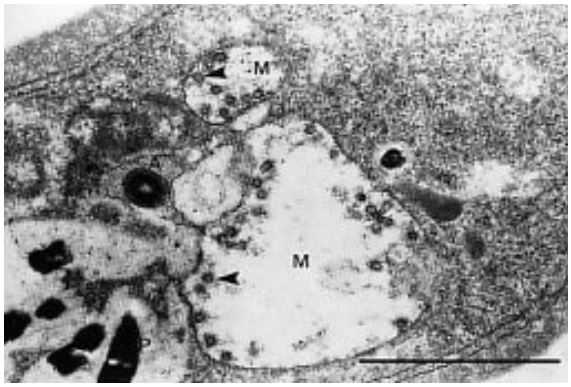

Fig 11 TEM of "Type III'mitochondria in a male gametocyte. An apicoplast is observed with a multi-membranous organelle containing electron-dense matrix and absence of internal cristae. The bar represents 1 µM. M, mitochondria; P, crystalline pigment. A, apicoplast.

Table 2. The mitochondrial oxygen consumption by *P. falciparum* at asexual and sexual stages of development.

Stages of parasite	Oxygen consumption [a] (nmol/min/10 [8] parasites)		
	Control	+Atovaquone [b]	+Cyanide [c]
Asexual stage	204±16	10±2	97±10
Sexual stage	242±20	126±14	30±4

[a] Values are means ±SD, taken from 3-4 separate experiments of the parasite preparations.
[b] Final concentration used was $1x10^{-6}$M atovaquone.
[c] Final concentration used was $1x10^{-3}$M potassium cyanide.

Table 3. The 50% effective concentrations (EC_{50}) of mitochondrial electron transport system and pyrimidine biosynthetic pathway inhibitors on parasite oxygen consumption.

Stages of parasite	EC_{50} (M)		
	Atovaquone	Cyanide	5-Fluor oorotate
Asexual stage	$5x10^{-8}$	$9x10^{-4}$	$1x10^{-7}$
Sexual stage	$9x10^{-7}$	$2x10^{-4}$	$5x10^{-7}$

(Tables 2 and 3). The antimalarial drug atovaquone is a mitochondrial inhibitor of the parasite electron transporting complex III (ubiquinol-cytochrome c reductase).[10,26-28] Furthermore, the atovaquone had moderate gametocytocidal activity with IC_{50} of $5x10^{-8}$ M, compared to its potent blood schizontocidal activity (IC_{50}=$5x10^{-10}$ M). It has been recently shown that atovaquone is indeed a gametocytocidal drug.[29] These lines of evidence would provide mitochondria in the sexual gametocytic stages as a possible chemotherapeutic target.

Our results suggest that the abundant mitochondria in the sexual gametocytic stage parasites were still in the underdeveloped forms but biochemically active at least with regards to oxygen consumption. The mitochondrial heterogeneity also suggests their role in energy production. Mitochondrial ATP synthetase inhibitors are reported to have antimalarial activity against the asexual growth of *P. falciparum* at micromolar concentrations.[8,25,30] Existence of ATP synthetase activity which is responsible for ATP production in the sexual gametocytic stage remains to be elucidated.

The findings on the relatively low oxygen consumption and reduced sensitivity to cyanide (Tables 2 and 3) suggest that *P. falciparum* operates either a branched chain respiratory pathway containing a non-cytochrome electron transport system with an alternative oxidase which is insensitive to cyanide[6,31] or a branched electron transport chain consisting of a specialized cytochrome system in which fumarate acts as an electron acceptor and is reduced to succinate by an NADH-dependent fumarate reductase.[3] The latter pathway has been described in the adult round worm *Ascaris suum* showing that ATP is anaerobically produced by substrate level phosphorylation in the branched electron transport pathway.[32]

More interestingly, 5-fluoroorotate is reported to be a potent inhibitor of pyrimidine biosynthetic pathway of *P.falciparum*.[19,33] It showed marked inhibitory effect on the oxygen consumption by both asexual and sexual stage parasites (Table 3), suggesting that the parasite in the sexual stage has linkage of the two metabolic pathways, pyrimidine biosynthesis and mitochondrial electron transport system, through dihydroorotate dehydrogenase. The association between the pyrimidine pathway and mitochondrial electron transport system in the asexual stage parasite has been confirmed.[5,9] It is then concluded that *P. falciparum* in both developmental stages have functional mitochondria that contribute significantly to *de novo* synthesis of pyrimidine and to the energy metabolism of the parasite.

Whether or not the mitochondrial heterogeneity observed in the parasites have functional significance must await study of their biochemistry and physiology, for instance, enzymatic activities of the mitochondrial electron transport chain in the sexual stage parasite, differences in the mechanism of energy metabolism, mitochondrial ATP synthetase, and role of oxygen tension on gametocytes circulating in human blood system.

ACKNOWLEDGEMENTS

We thank S Vettchagarun and D Burat for their dedicated technical assistance with EM techniques and some experiments on oxygen uptake. The parasites T_9 and KT_3 isolates were kindly provided by S Thaitong of Chulalongkorn University and P Petmitr of Mahidol University, respectively. This work was supported by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. J Krungkrai is a career development award recipient from the National Science and Technology Development Agency of Thailand.

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