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Degenerative Changes in Morphology of *Plasmodium falciparum* Induced by Artemether *In vitro*

TOSHIRO ONO[1], MEIJI ARAI[1], KUNIO SHIMONO[1], TOSHIKI AJI[1], NOBUO OHTA[1] AND AKIRA ISHII[2]

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Abstract

Antimalarial action of methyl-dihydro-artemisinin (artemether), the methoxy derivative of qinghaosu, was studied ultrastructurally in human erythrocytes infected with *Plasmodium falciparun in vitro*. More than 25 nM of artemether effectively inhibited the growth of the parasites. Ultrastructural observations revealed much progressed disintegration in the parasites as early as 5 hr after the exposure of artemether at concentrations of 50 to 100 nM, showing ribosome clumpling, a dilated nuclear envelope and endoplasmic reticulum, a disintegrated food vacuole, and swollen mitochondria with markedly conspicuous cisternae. Focal and entire disruptions of membranous structures of the parasites was also found. The results indicate that the site of the action of artemether is the membranous structures, suggesting the responsibility of lipid peroxidation process.

Key words: *Plasmodium falciparum,* Malaria, Artemether, Ultrastructure

Introduction

The fast growing prevalence of drug-resistant *Plasmodium falciparum* maralia is now a major health problem in the developing countries. Continuing efforts to develop new therapeutic agents for control of it are of considerable current interest (Peters, 1980; Wyler, 1983). Qinghaosu (artemisinin) recently isolated from the traditional Chinese herb, *Artemesia annua L.*, was shown to have antimalarial activity (Qinghaosu Antimalaria Coordinating Research Group, 1979), by a function as an oxidant (Krungkai and Yuthavong, 1987; Scott *et al.*, 1989). Plasmodicidal effects of qinghaosu and its methoxy, ethoxy and hemisuccinate derivatives have been confirmed in experimental studies (Gu *et al.*, 1983; Li *et al.*, 1983; Ye *et al.*, 1987) and in clinical trials (Jiang *et al.*, 1982) against both chloroquine-sensitive and -resistant strains of *P. falciparum*. However, little studies are available on the ultrastructural changes of *P. falciparum* induced by qinghaosu and its derivatives.

In the present study, we performed careful ultrastructural observations on *P. falciparum* after *in vitro* exposure to an oil-soluble methoxy derivative of methyl-dihydro-artemisinin (artemether), which is intramuscularly injectable and shown to be more effective than qinghaosu (Gu *et al.*, 1983), to examine the site of action of the drug and morphological changes in the parasites.

[1]*Department of Parasitology, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700, Japan.*
[2]*Department of Parasitology, National Institute of Health, Kamiosaki, Shinagawa-ku, Tokyo 141, Japan.*
小野俊朗　新井明治　下野國夫　安治敏樹　太田伸生（岡山大学医学部寄生虫学教室）
石井　明（国立予防衛生研究所寄生虫部）

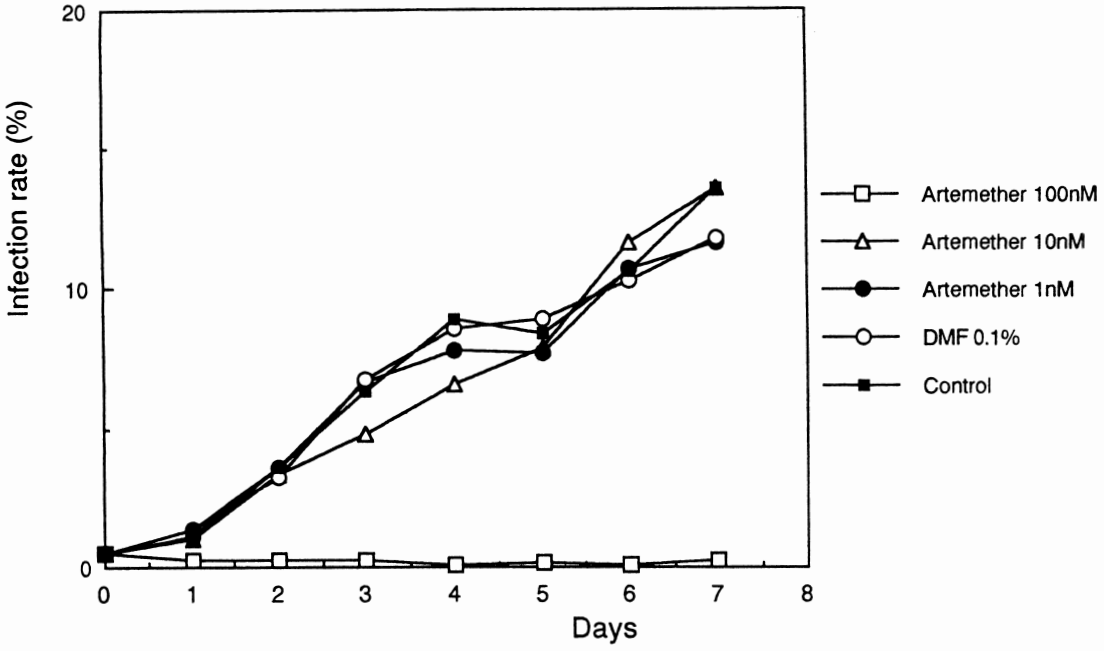
Materials and Methods

Parasites

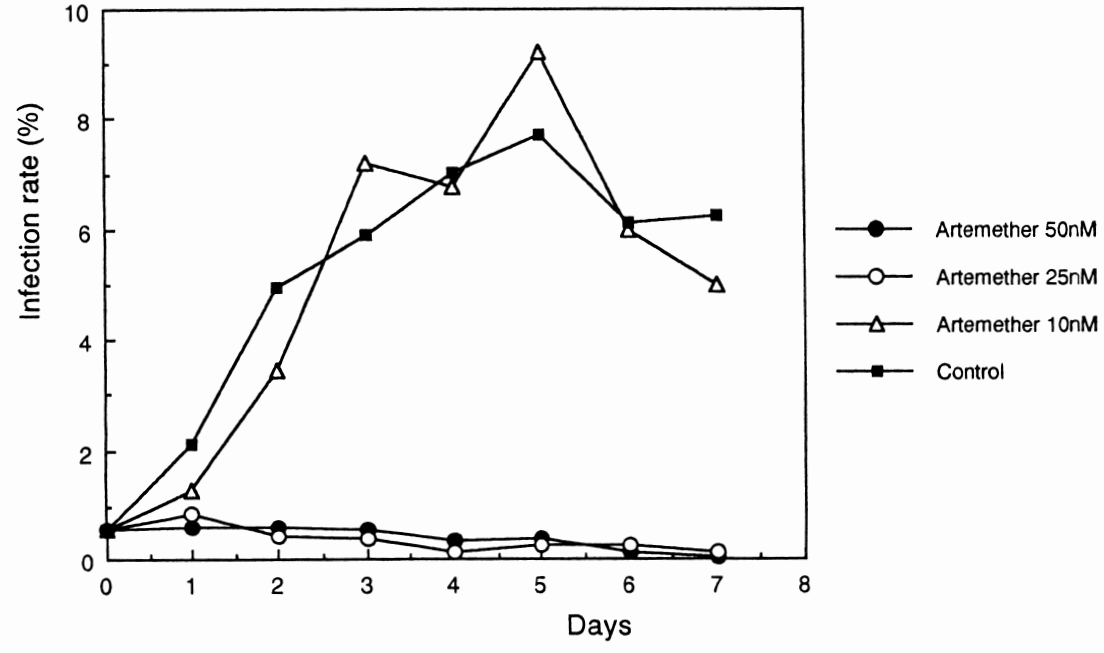
FCR-3 strain of *Plasmodium falciparum,* which is resistant for chloroquine, has been maintained in our laboratory for years (Doi *et al.*, 1988). Parasites were cultured by the method of Jensen and Trager (1977) in RPMI 1640 medium (Gibco) supplemented with 25 mmol/l HEPES (Sigma), 2 g/l $NaHCO_3$, 25 mg/l gentamicin (Schering-plough) and 10% human serum type A.

Artemether

Artemether was purchased from Kunming pharmaceutical Co. (Yunnan, China). It was



(a)



(b)

Fig. 1 Effect of artemether on the growth of *P. falciparum*. Parasites were cultured in RPMI growth medium containing various concentrations (1, 10, and 100 nM in (a), and 10, 25, and 50 nM in (b)) of artemether. These concentrations were maintained throughout the 7 days of culture by changing the medium every day. The results are the means of triplicate measurements.

dissolved and diluted to 100 μmol/l in dimethylformamide (DMF) and kept at −20°C until use.

Cultivation of P. falciparum with artemether

Parasites were synchronized to "early ring" enriched stage with sorbitol treatment (Lambros and Vanderberg, 1979). After synchronization, they were diluted to a final parasitemia to 0.5% hematocrit by using type A human red blood cells, and placed in 24 well tissue culture plates. Artemether diluted to concentrations ranging from 1 to 100 nmol/l with RPMI 1640 medium was added to each well, and the plates were incubated at 37°C for 7 days. Medium with or without the drug was changed daily. Air-dried thin smears of infected erythrocytes were fixed in methanol, stained with Giemsa, and examined under light microscopy. Infection rate was determined by microscopical counts of the number of parasites in a minimum of 2,000 erythrocytes. All experiments were repeated at least three times.

Electron microscopy

Cultures were fixed in 2% glutaraldehyde-2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 1 hr. They were then post-fixed in 1% OsO_4 in 0.1M cacodylate buffer at 4°C for 1 hr, dehydrated through graded ethanol to propylene oxide, and embedded in epon-812 resin. Ultra-thin sections of silver color were cut with a Reichert OmU-2 ultramicrotome, and observed under a Hitachi electron microscope, H-700, after staining by uranyl acetate and lead citrate.

Results

Effect of artemether on the growth of P. falciparum

The *in vitro* effect of artemether on the growth of *P. falciparum* is shown in Fig. 1a. A hundred nanomolar artemether completely inhibited parasite growth in less than 1 day. A steady increase in number of parasitized cells was observed on both concentrations of 1 and 10 nM artemether treated cultures. Treatment of 0.1% DMF corresponding to the concentration of solvent also showed a similar growth curve as that of untreated control cultures. On the basis of the similarities, there is no deleterious effect on the parasite growth. To examine the minimum concentration of the drug required for killing of parasites, another series of experiment was performed by treating the cultures with 10, 25 and 50 nM artemether. As shown in Fig. 1b, 25 nM artemether achieved nearly total inhibitory effect on the parasite growth.

Light microscopy

Light microscopic observations on thin smears

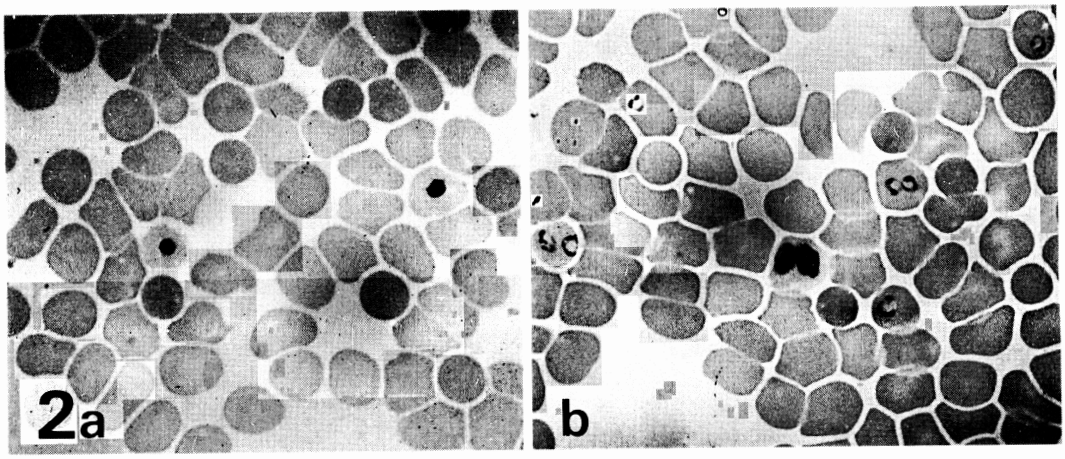


Fig. 2 Light micrograph of thin smears of *P. falciparum*-infected erythrocytes. (a) One day after the exposure of 100 nM artemether. Most parasites show pyknotic nuclei. (b) Untreated control cells. Cells were stained with Giemsa. ×880.

stained with Giemsa revealed morphological changes as early as 5 hr after the exposure of artemether. Most of parasites were shrunken with pyknotic nuclei 1 day after the exposure at a concentration of both 50 and 100 nM, clearly showing necrotic alterations (Fig. 2). Twenty five nanomolar artemether caused a moderate morphological effect, with only half of parasites showing such drastic alterations on day 1 (data not shown).

Ultrastructural changes of P. falciparum

Electron microscope observations of *P. falciparum* in erythrocytes at specific intervals after the exposure of 100 nM artemether revealed degenerative changes of the parasites. The parasites showed electron dense cytoplasm and an increased number of free ribosomes as early as 1 hr of the exposure (Fig. 4). Following exposure time, 5–24 hr, much progressed disintegration was found in the parasites. The parasites showed marked ribosome clumpling, a dilated nuclear envelope and endoplasmic reticulum, a disintegrated food vacuole with pigment scattering. They also contained numerous small cytoplasmic vacuoles and swollen mitochondria with markedly conspicuous cisternae. They also showed much electron dense cytoplasms and appeared large masses within electron lucent erythrocytes (Fig. 5). Focal disruptions of plasma membrane and other cytoplasmic vacuoles of the parasites were occasionally found within 5 hr of the exposure (Fig. 6). And host erythrocytes had much electron lucent cytoplasm. In addition, at 24 hr of the exposure the infected erythrocytes entirely lost their cytoplasmic contents. Much swollen and disintegrated parasites were found within the erythrocyte ghost, clearly showing their overt necrosis (Fig. 7).

Discussion

The present findings clearly show that more than 25 nM of artemether inhibited *P. falciparum* growth *in vitro*. Morphological observations revealed injury to the membranous structures of the parasites as early as 5 hr of the exposure, showing an extensive and necrotic damage to the

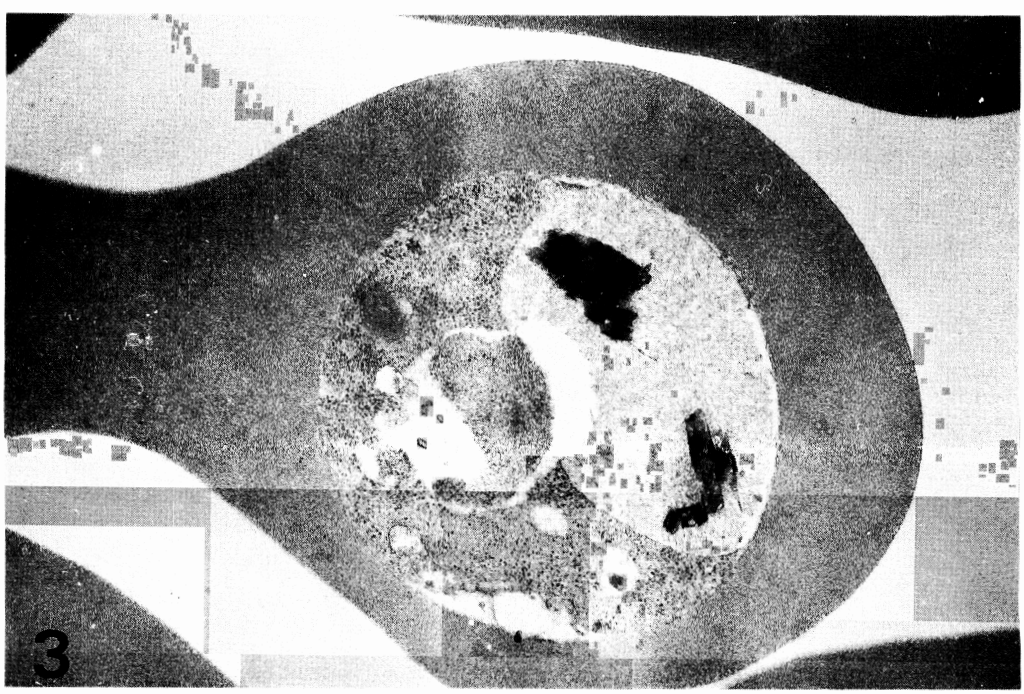


Fig. 3 Electron micrograph of normal *P. falciparum*. ×24,000.

Fig. 4 Electron micrograph of *P. falciparum* fixed 1 hr after the exposure of 100 nM artemether. ×45,000.

parasites.

Qinghaosu and its derivatives have an unique group, bridged endoperoxides, by which they may function as an efficient initiator of membrane lipid peroxidation in cells (Scott *et al.*, 1989). Because its deoxy derivative, which lacks the dioxygen bridge, has no antimalarial activity (Brossi *et al.*, 1988). In addition, other oxidative pharmacologic drugs such as primaquine and alloxan also have antimalarial activity (Summerfield and Tudhope, 1978; Clark and Hunt, 1983).

Jiang *et al.* (1985) showed that qinghaosu primarily caused swelling of mitochondria of *P. inui* after an administration of the drug to the host animals, and suggested direct inhibition of oxidative phosphorylation of the parasite mitochondria. Such structural changes was similar to that of primaquine (Beaudoin and Aikawa, 1968). Jiang *et al.* (1985) also indicated that several hours of drug exposure required to injury to the membranous structures of the parasites. We found similar mitochondrial changes in *P. falciparum*. Outstanding and widespread injury to the membranous structures of both the parasites and infected erythrocytes was also observed as early as 5 hr of the artemether exposure. Our results are well consistent with the previous studies (Jiang *et al.*, 1985). Although we could not obtain any direct evidence, these morphological findings support the role of lipid peroxidation process in the antimalarial action, which is mediated by active oxygen species derived from the endoperoxide in the molecules.

Selective destruction of the parasites as well as the infected erythrocytes is another characteristic finding. No morphological changes was found in the uninfected erythrocytes throughout the examination period. It might be due to the biochemical and morphological alterations in the infected erythrocyte membrane, including changes in permiability of molecules (Ginsburg *et al.*, 1985; Tanabe, 1990), and the osmotic fragility (Clark *et al.*, 1983; Cranston *et al.*, 1984), a rearrangement of intramembranous particles (Aikawa *et al.*, 1978), and disorder in phospholipid packing of the bilayer (Taraschi *et al.*, 1986; Schwartz *et al.*, 1987). Furthermore, qinghaosu was shown to be selectively con-

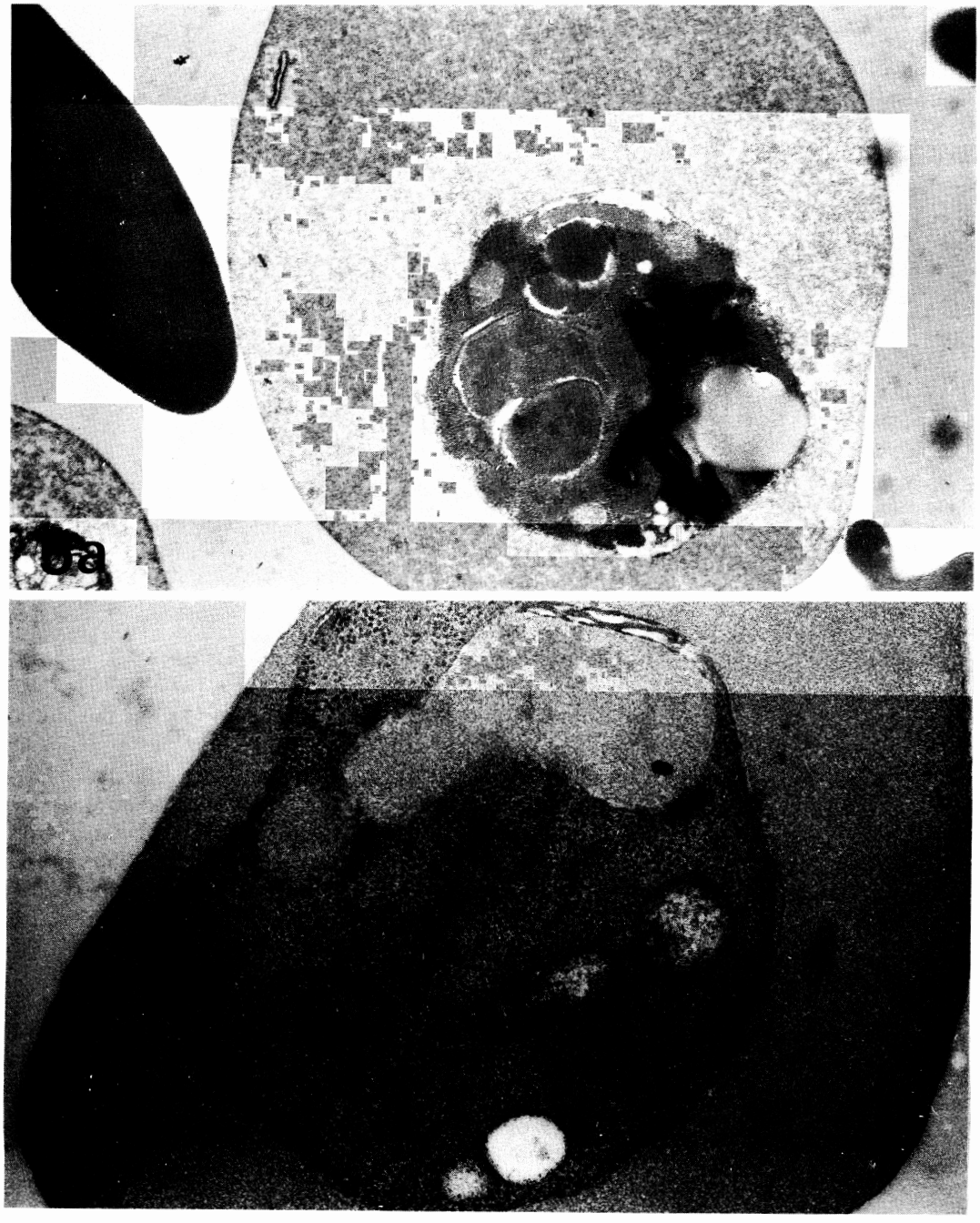


Fig. 5 Electron micrographs of *P. falciparum* fixed 5 hr (a) and 24 hr (b) after the exposure of 100 nM artemether. a), ×18,000; b), ×36,000.

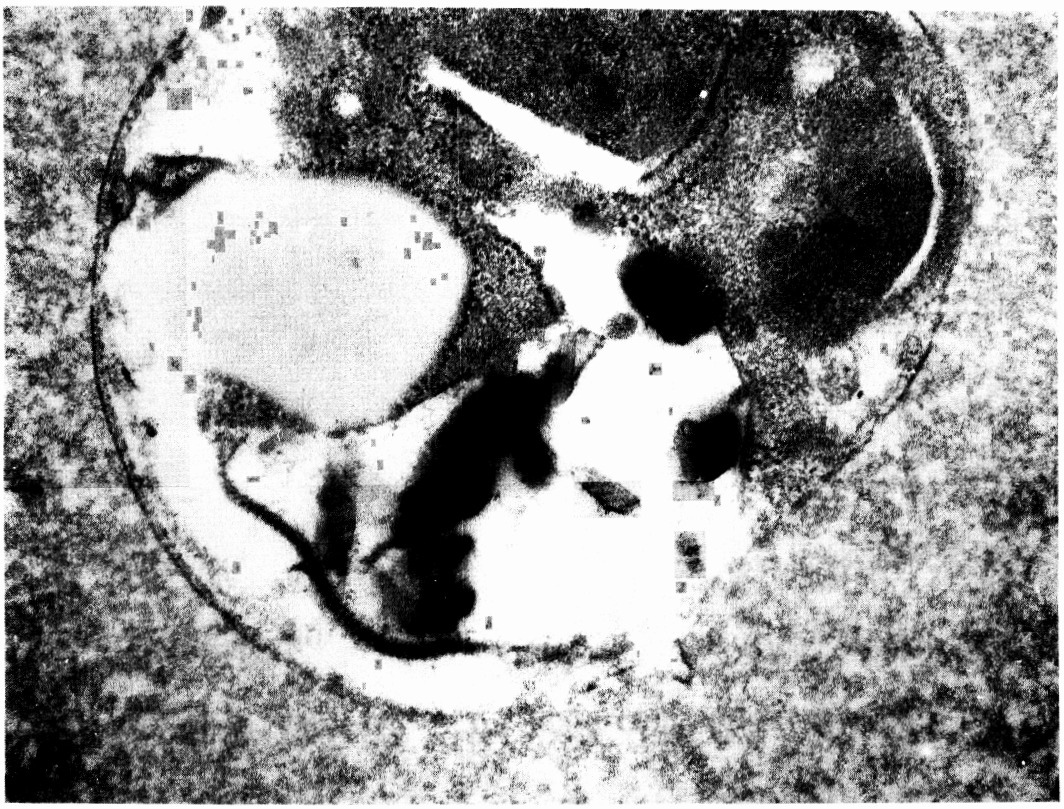


Fig. 6 Electron micrograph of *P. falciparum* fixed 5 hr after the exposure of 100 nM artemether. ×45,000.

centrated in infected erythrocytes (Gu *et al.*, 1984). The extreme oxidant-sensitivity of the malarial parasites (Wozencraft, 1986) and exceptional oxidant resistance of the normal erythrocytes (Stocks and Dormandy, 1971) may also account for this discrepancy of the drug sensitivity.

In conclusion, the results of our experiments show the artemether causes an extensive injury to the cellular and subcellular membranous structures of *P. falciparum,* suggesting the responsibility of the lipid peroxidation process. Although there is substantial evidence for lipid peroxidation after in vitro exposure of *Plasmodium*-infected erythrocytes to oxidants (Clark *et al.*, 1984; Stocker *et al.*, 1985), a detailed biochemical studies are needed to elucidate the active oxygen species responsible for the antimalarial action.

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Fig. 7 Electron micrograph of *P. falciparum* fixed 24 hr after the exposure of 100 nM artemether. Arrows: erythrocyte membrane. ×36,000.

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