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Electron Microscopy of Intracellular Protozoa

Annual Report

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Verapamil, a calcium antagonist, has recently been shown to reverse chloroquine resistance in malarial parasites in vitro. In collaboration with Major Milhous and his associates, we studied morphological changes associated with this phenomenon using chloroquine-sensitive and -resistant clones of P. falciparum. While the administration of 6.3×10^{-8} M chloroquine had little morphological effect on the chloroquine-resistant strain, the combination of chloroquine and verapamil resulted in typical chloroquine-related food vacuolar swelling with increased amounts of granular matrix. Secondary morphological changes included degeneration of nuclei, mitochondria and other organelles. These effects appeared similar to those in the

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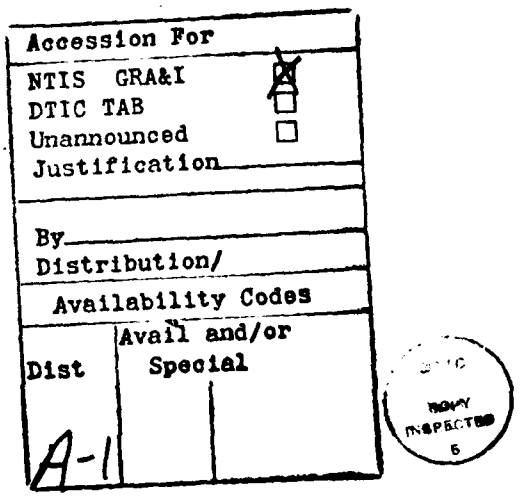
19. Abstract (continued)

chloroquine-sensitive strain of P. falciparum treated with chloroquine alone or with the chloroquine/verapamil combination. Therefore, our results support the suggestion that verapamil reverses chloroquine resistance in malarial parasites.

We also performed an experiment on ultrastructural localization of protective antigens of P. falciparum by postembedding immunoelectron microscopy in collaboration with Col. J. Chulay of WRAIR. The 101 kDa antigen recognized by monoclonal 3D5 is synthesized by mature trophozoites and young schizonts and appears in the culture medium when schizont rupture occurs in normal culture medium. Immunofluorescence with MAb 3D5 gave a grape-like pattern of rimmed fluorescence around merozoites contained within mature schizonts. Post embedding immunoelectron microscopy indicated that the antigen recognized by MAb 3D5 was present at the surface of schizonts within electron-dense material in the parasitophorous vacuole and at the surface of individual merozoites within schizont-infected cells.

Foreword

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).



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Ultrastructural Study of the Effects of

Chloroquine and Verapamil on *Plasmodium falciparum*

Abstract

Verapamil, a calcium antagonist, has recently been shown to reverse chloroquine resistance in malarial parasites in vitro. We report the first ultrastructural morphological changes associated with this phenomenon using chloroquine-sensitive and -resistant clones of P. falciparum. While the administration of 6.3×10^{-8} M chloroquine had little morphological effect on the chloroquine-resistant strain, the combination of chloroquine and verapamil resulted in typical chloroquine-related food vacuolar swelling with increased amounts of granular matrix. Secondary morphological changes included degeneration of nuclei, mitochondria and other organelles. These effects appeared similar to those in the chloroquine-sensitive strain of P. falciparum treated with chloroquine alone or with the chloroquine/verapamil combination. Furthermore mild food vacuolar changes were seen in a small number of parasites (from both chloroquine sensitive and resistant groups) exposed to high concentrations (1×10^{-4} M) verapamil alone.

Introduction

An escalating problem in the treatment of malaria in the last 25 years has been the emergence in many parts of the world of malarial parasites that are resistant to multiple differently structured antimalarial drugs[1]. With the induction of resistance to one antimalarial drug *in vitro*, there have been concomitant decreases in susceptibilities to drugs of different chemical classes. This multi-drug resistance appears similar to the multiple drug resistance (MDR) phenomenon which occurs in neoplastic cells[2]. Verapamil, a calcium channel blocking drug, used mainly as a coronary vasodilator, has been shown to reverse MDR in cultured cancer cells[2-4]. Recently *in vitro* biochemical studies of *Plasmodium falciparum* have shown similar reversal of chloroquine resistance after administration of verapamil[5]. The aim of our present study was to document morphological and physiological changes occurring within chloroquine-sensitive and -resistant parasites treated with the chloroquine alone and in combination with verapamil.

Materials and Methods

This *in vitro* study was performed with cloned chloroquine-sensitive (CS) (West African D-6) and chloroquine-resistant (CR) (W2 Indochina) *P. falciparum* strains[6]. Control cultures were maintained in parallel with cultures incubated in medium containing chloroquine (6.3×10^{-8}M) or verapamil (1.0×10^{-4} or 1.8×10^{-6}M) alone and chloroquine 6.3×10^{-8}M plus 1.8×10^{-6}M verapamil. All cultures were incubated for 24 hours after which each culture was centrifuged with a discontinuous percoll gradient[7,8] to concentrate the parasites. Samples were fixed in 2.5% glutaraldehyde solution with 4% sucrose and 0.05M phosphate buffer (pH 7.4). They were washed in the same buffer and post-fixed in 1% osmium tetroxide before being dehydrated and embedded in Epon 812. The resulting blocks were sectioned with a Porter-Blum MT-2

ultramicrotome using a Dupont diamond knife. Semi-thin sections were prepared and stained with methylene blue. Finally thin sections, mounted on 200-mesh nickel grids and stained with 1% uranyl acetate and lead citrate, were examined with a Philips 201 electron microscope. (Simultaneous in vitro concentration response controls were conducted using the method of Martin et al., 1987[5] to determine intrinsic antimalarial drug effects or uptake of tritiated hypoxanthine).

Results

Ultrastructural examination of the different groups of cultures revealed the following. Of those exposed to chloroquine alone, the CS strain showed changes previously described with administration of this drug[9-12]. Food vacuole enlargement with increased granular matrix material was seen (Fig. 1). The pigment particles became focally clumped. In some parasites, the food vacuole membrane disintegrated and the pigment granules lay free within the cytoplasm along with empty vesicles and myelin figures (Fig. 1). Some nuclei became more densely stained and coarsely clumped. The cytoplasm showed ribosomal aggregation as well as patchy loss. Mitochondria appeared slightly swollen and rough endoplasmic reticulum became difficult to identify. In some parasites, the plasma membrane had focally disintegrated and the parasitic contour was irregular.

Electron microscopy of the CR clone of P. falciparum treated with chloroquine alone did not reveal the above changes, as was the case with both the CS and CR control cultures. Food vacuoles were normal or only minimally enlarged and other cell constituents appeared normal (Fig. 2). Minimal food vacuolar enlargement is sometimes recognized in P. falciparum grown in vitro.

Both chloroquine sensitive and resistant cultures exposed to a small dose of verapamil (1.8×10^{-6}M) showed a few parasites with similar early food

vacuolar swelling and increased granular matrix but without any of the nuclear or other cytoplasmic changes described with chloroquine (Fig 3).

When the larger dose of verapamil ($1.0x10^{-4}M$) was administered, significant alterations in the food vacuoles and other cytoplasmic organelles were observed. Food vacuoles were moderately to severely enlarged with focally reduced (Fig. 4) and dispersed crystalline pigment granules and occasional membranous debris, however no disintegration of food vacuole membranes was observed. Mitochondria appeared somewhat swollen with an increase in electron lucent matrix, with occasional myelin figures and membranous debris. Rare degenerate parasites were observed. Some early merozoites showed mitochondrial changes and cytoplasmic vacuolization.

In cultures exposed to the combination of chloroquine $6.3x10^{-8}M$ plus verapamil $1.8x10^{-6}M$ the CS clone showed food vacuolar swelling, irregular contours and accumulation of electron lucent matrix and membranous debris. Pigment granules were clumped and focally decreased in number (Fig. 5a). Mitochondrial swelling, ribosomal clumping and focal cytoplasmic vacuolization were also observed. Significant numbers of organisms showed advanced degenerative changes appearing markedly dense and containing large numbers of small round empty vacuoles and dense granules of various size (Fig. 5b). Rupture of cell membranes and desintegration of other organisms was also noted.

The CR clone treated with the same chloroquine/verapamil combination showed similar changes to the CS clone with very prominent food vacuolar changes but less advanced degeneration of entire parasites (Fig. 6). Food vacuoles were noted to be markedly dilated with dispersion of pigment granules and many large empty membranous vesicles. Rupture of the membrane surrounding some food vacuoles with spillage of their contents into the parasite cytoplasm

was occasionally seen. There was mild mitochondrial enlargement and focal early ribosomal loss.

Simultaneous in vitro testing of chloroquine alone or in combination with verapamil revealed a three fold increase in drug effect (IC_{50} decreased from 71.2×10^{-9} to 24.3×10^{-9}M) against the CR clone with no significant changes in the CS clone.

Discussion

The administration of chloroquine to drug sensitive organisms resulted in the typical food vacuolar alterations within the parasites. The drug-resistant clone failed to reveal any comparable changes. When a small dose of verapamil was added to chloroquine, the drug-resistant clone exhibited alterations morphologically similar to those of the sensitive strain, suggesting that reversal of drug resistance had occurred. The alterations in the CR parasites appeared slightly less advanced than those observed in the CS clone treated with both drugs.

The prominent alterations of food vacuoles noted in the CR strain of P. falciparum after incubation with chloroquine/low dose verapamil, which were absent in parasite incubated with chloroquine alone, suggests that verapamil is able to reverse chloroquine resistance in some manner. In vitro studies suggest that the basis of drug resistance in cancer cells is related to the enhanced active elimination of the chemotherapeutic agent from the cells' cytoplasm thus protecting them from rising drug concentrations liable to cause toxic cell damage[13]. Blocking of this accelerated elimination process results in reversal of drug resistance in the cells. Verapamil is widely known as a cardiac-active calcium channel blocking drug but has recently been shown to be capable of reversing drug resistance in cultured cancer cells by interfering

with the process of accelerated elimination of the chemotherapeutic agent from the cells[2,3].

It has previously been demonstrated that the antimalarial activity of chloroquine and other quinoline-containing antimalarial drugs is related to the concentration of the drug within the parasite, and especially within the food vacuoles[14,15]. Chloroquine resistance in P. falciparum has also been related to decreased drug accumulation in chloroquine resistant parasites. Other biochemical studies[5] measuring the rate of inhibition of asynchronous parasitic growth by radiolabeled hypoxanthine incorporation techniques showed that verapamil is capable of exerting a marked potentiation of drug effect when used with chloroquine against the drug-resistant strain of P. falciparum. No potentiation of drug effect was detected in the chloroquine sensitive organisms treated similarly. Verapamil administered alone did appear to have mild intrinsic antimalarial properties in vitro, but at very high levels which cannot be clinically achieved in vivo and exhibit significant toxicity. Verapamil alone is at least 1000 times less potent than chloroquine or mefloquine. This action, although of no clinical significance, was also confirmed ultrastructurally in the present study by the presence of food vacuolar changes, especially in the organisms exposed to the higher dose of verapamil.

With improved pharmacokinetics and reductions in toxicity[18], the clinical utility of companion drugs with verapamil-like properties may substantially improve the management of both drug resistant neoplastic and infectious diseases.

TABLE I SUMMARY OF FOOD VACUOLE CHANGES

GROUP	CS STRAIN	CR STRAIN
CONTROL	0	0
Chloroquine (6.3×10^{-8}M) alone	+ + +	0
Verapamil (1.8×10^{-6}M) alone	0/+	0/+
Verapamil (1.0×10^{-4}M) alone	+ to + +	+ to + +
Chloroquine (6.3×10^{-8}M) plus Verapamil (1.8×10^{-6}M)	+ + +	+ + +

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Figure Legends

Fig. 1 CS P. falciparum strain treated with chloroquine alone. Marked enlargement of food vacuoles (Fv) containing pigment (P) granules and granular matrix material is present. Degenerate membranous changes are also seen (arrow). X 21,000.

Fig. 2 CR P. falciparum strain exposed to chloroquine only. Food vacuoles (Fv) appear compact with no evidence of swelling. The clear spaces seen in the food vacuoles are the result of the extraction of malaria pigment by the high pH of the staining agent, lead citrate (arrows). X 33,000. Inset: Normal food vacuole (Fv) of control P. falciparum. X 26,000.

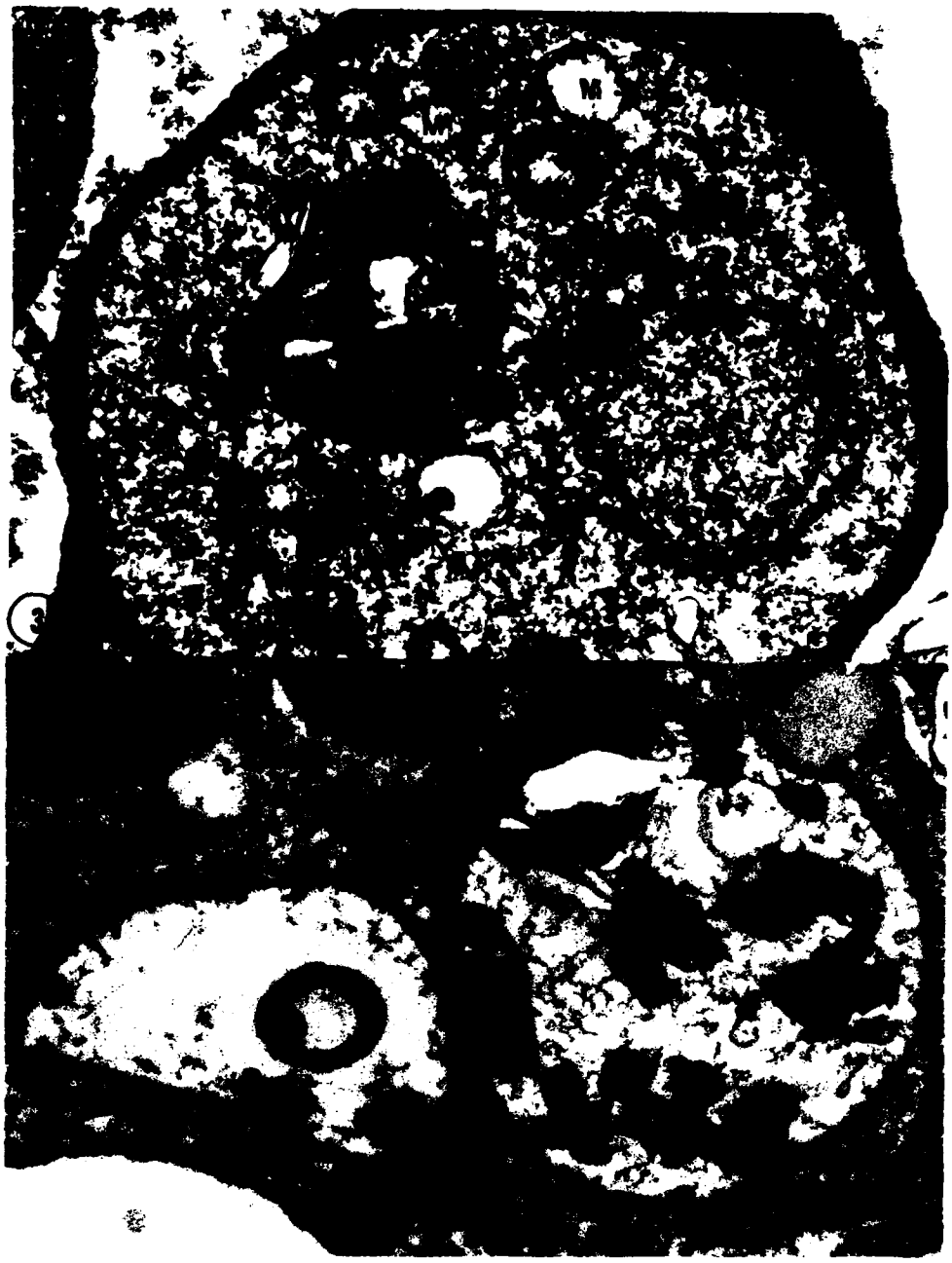
Fig. 3 CS P. falciparum treated with small dose of verapamil. Parasite showing normal food vacuole (Fv) and mitochondria (M). X 40,000.

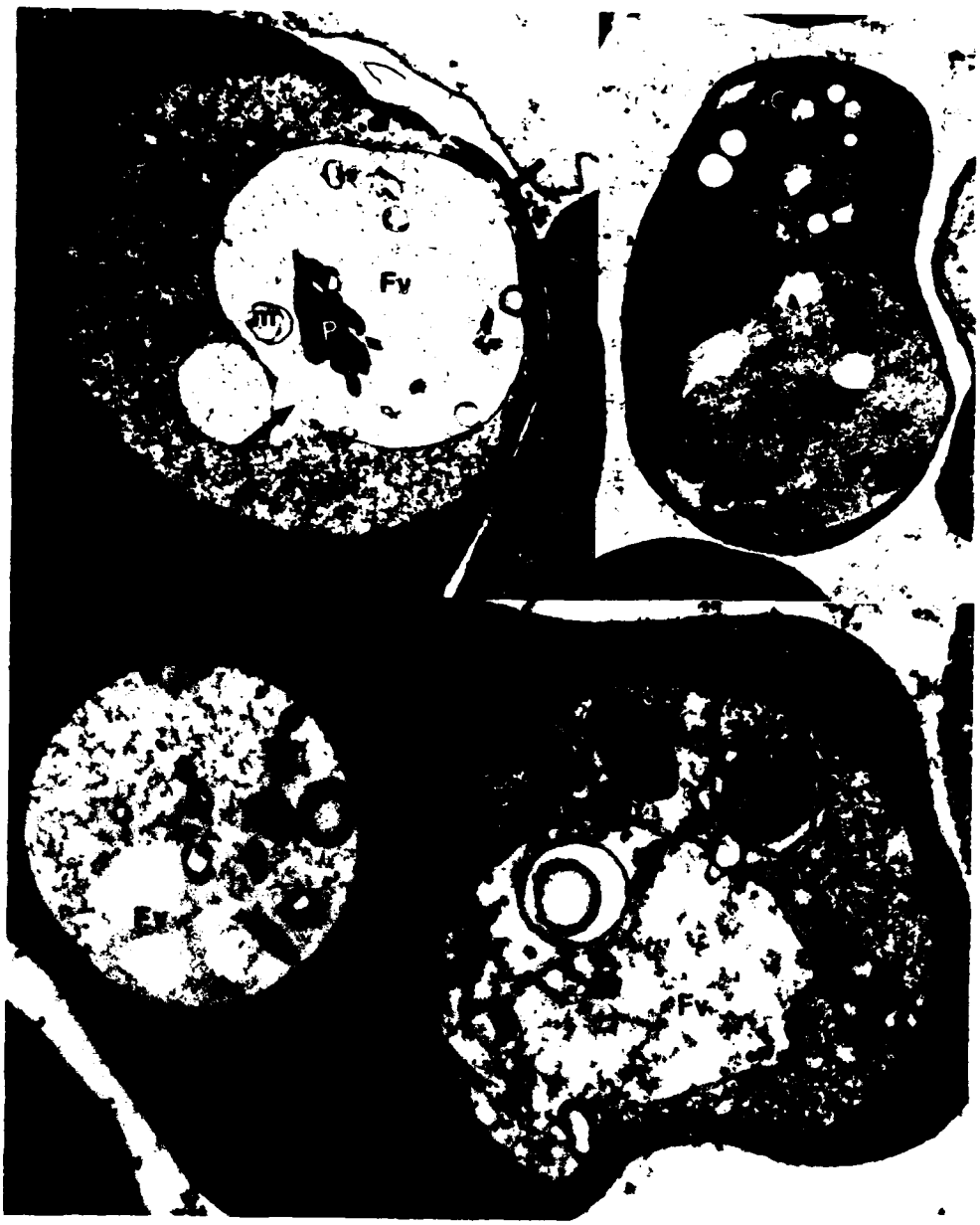
Fig. 4 CS P. falciparum strain exposed to large dose of verapamil. Moderate food vacuole (Fv) swelling accompanied by marked mitochondrial enlargement (M). X 52,000.

Fig. 5 CS P. falciparum strain treated with chloroquine/verapamil combination. a) Food vacuole (Fv) showing enlargement and increased granular matrix, pigment granules (P) and membranous structure (m). Focal degenerations of the food vacuole membrane are noted (M) X 17,000. b) Erythrocyte with 2 degenerating parasites, one of which has become dense and contains vacuoles (V) and granules (G). The other is relatively intact and shows budding merozoites with rhoptries (R). X 10,300.

Fig. 6 CR strain exposed to chloroquine plus verapamil. Advanced food vacuolar swelling (Fv) with interruption of food vacuole membrane (arrows). X 26,000.


Fv
Fv


M


Fv
P
Fv
Fv

Morphological Effects of Pyronaridine

on Malarial Parasites

Abstract

The ultrastructural changes caused by the new antimalarial drug, pyronaridine, were investigated using mice infected with erythrocytic forms of Plasmodium berghei and Plasmodium falciparum cultivated in vitro in human erythrocytes. The first changes observed in both parasites after exposure to pyronaridine occurred in the food vacuoles. This suggests that the target organelle of this drug may be the food vacuole of malarial parasites. In addition, rapid alterations were also noted within the pellicular complex of both plasmodia.

Introduction

The widespread development of chloroquine-resistant malarial strains has resulted in renewed urgency in the search for alternative effective antimalarial drugs. Pyronaridine phosphate, coded 7351, which was synthesized in China in 1979, is a new schizonticidal agent active against the erythrocytic stages of malarial parasites.[1] The results of animal experiments and clinical trials indicate that pyronaridine is highly effective with low toxicity and no cross resistance with chloroquine in the treatment of falciparum and vivax forms of malaria.[2-5] It has also been found useful in treating infections due to multiple drug-resistant P. falciparum, where chloroquine combined with either pyrimethamine, sulfamido-derivatives or mefloquine has been ineffective.[6] Administration is simplified by the flexibility of being able to use either oral, intramuscular or intravenous routes.

In order to try to determine the mode of action of pyronaridine, we have undertaken an ultrastructural study of the drug's morphological effects on P. falciparum cultured in vitro in human erythrocytes and P. berghei in vivo in mice.

Materials and Methods

P. berghei:

Fourteen SWR/J mice (Jackson Laboratory, Maine), each weighing an average of 18 grams, were inoculated intraperitoneally with erythrocytic forms of the B. strain of Plasmodium berghei. Daily Giemsa-stained blood smears were examined until parasitemia reached about 35%. Two control mice were designated and the remaining 12 treated with a single therapeutic dose (20 mg base/kg body weight) of pyronaridine phosphate (Institute of Parasitic Diseases, Chinese Academy of Preventive Medicine, Shanghai) dissolved in distilled water and administered by intraperitoneal injection. ~~The ED_{50} of the drug has been determined to be 20 mg/kg~~[1-4], while that of chloroquine is ~~40 mg/kg~~. Blood samples for electron and light microscopy were collected at 0.5, 2, 4, 8, 16 and 24 hours after drug administration. For electron microscopy, 0.5-1 ml blood samples were fixed in 2.5% glutaraldehyde solution with 0.05 M phosphate buffer (pH 7.3) and sucrose at 4°C for 2 hrs and then processed routinely. The resulting blocks were cut with a Porter-Blum MT-2 ultramicrotome using a Dupont diamond knife. Ultrathin sections were mounted on 200-mesh nickel grids, stained with 1% uranyl acetate and lead citrate and then examined with a JEOL 100 CX electron microscope.

P. falciparum:

Plasmodium falciparum, NIH-7G8, was grown in human erythrocytes in vitro according to the method of Trager and Jensen[7]. When 10-15% of the erythrocytes were infected, the cultures were exposed to two different dosages

of pyronaridine phosphate, at concentrations of 23 x 10^{-9}M (ED_{50}) (unpublished data) and 10^{-6}M (corresponding to therapeutic concentration of chloroquine). The drug was first dissolved in a small quantity of distilled water and subsequently diluted to the appropriate concentration with RPMI 1640 medium. Blood samples were obtained at 0.5, 1, 2, 4 and 8 hours respectively after exposure to pyronaridine. The cell suspensions were centrifuged at 350 g for 5 minutes at 35°C, the supernatant discarded, and the pellet resuspended in fixative and processed for electron microscopy, as previously described. Untreated cultures were used as controls.

Results

P. berghei

Electron microscopy revealed the following sequential changes in the morphology of the erythrocytic forms of Plasmodium berghei after the administration of pyronaridine.

The most distinctive changes occurred in the food vacuoles. Trophozoites and young schizonts from untreated mice demonstrated small peripherally located food vacuoles limited by a single unit membrane and containing a few malarial pigment particles. Thirty minutes after the administration of pyronaridine, the food vacuoles began to aggregate and in some cases fused into one or two large vacuoles containing many pigment particles and some small single membrane-bound vesicles (Fig. 1). Many of the vesicles appeared to be filled with electron dense material similar to erythrocyte cytoplasm. Enlarged food vacuoles were identified in almost all

the trophozoites and young schizonts by two hours after drug administration. Also seen within the fused food vacuoles were membranous debris and myelin figures, some of which surrounded the small vesicles. As the food vacuolar matrix became more coarsely granular, the amount of malarial pigment decreased. The food vacuole alterations appeared fully developed by two hours after drug administration. At 24 hours only occasional trophozoites remained showing large vacuoles filled with small vesicles but devoid of pigment (Fig. 5).

Another morphologic change which appeared in the pellicular complexes of trophozoites, schizonts and gametocytes, consisted of the formation of multilamellate whorls and swelling of the pellicular complexes. Although this alteration was detected in only a few parasites at 30 minutes after drug administration, it became more widespread and pronounced over the next 3 1/2 hours (Figs. 1, 3, 4).

Starting after about 4 hours and following the food vacuolar and pellicular complex changes, secondary effects on other organelles were identified. Mitochondria became swollen and showed electron dense granules and fine fibrils within their matrix. Mild dilatation of the endoplasmic reticulum was seen. There were focal loss of ribosomes and the appearance of fine fibrillar material, multilamellate membranes and myelin figures within the parasitic cytoplasm. Nuclear borders became ill-defined and scattered degenerate parasites were identified.

Eight hours after pyronaridine administration, most parasites were markedly degenerate with some showing dense amorphous or fibrillar material

replacing recognizable organelles. By 16 to 24 hours after administration of the drug, only totally degenerate parasites and myelin figures were seen in the host cell cytoplasm. Light microscopic examination revealed only scattered small dense parasitic remnants without recognizable schizonts suggesting that clearing of parasites was occurring.

P. falciparum

Comparisons between untreated in vitro cultures of P. falciparum[8] and those treated with pyronaridine revealed significant ultrastructural changes in food vacuoles within 30 minutes after drug exposure (Fig. 6). The alterations were similar to those which occurred in P. berghei and included marked enlargement of the central food vacuole and the formation of intravacuolar vesicles and membranous whorls (Figs. 6-7). The number of altered food vacuoles and the severity of the changes progressed with time and increased dosage of pyronaridine.

The pellicular complex alterations in the in vitro P. falciparum organisms resembled those seen in P. berghei in vivo. These changes were first detected at 30 minutes after drug administration and developed progressively. Both swelling of pellicular complexes and the formation of multilamellate whorls were detected. Four hours after exposure to pyronaridine, the parasites showed other secondary changes such as dilatation of endoplasmic reticulum and degeneration of ribosomes and nuclear detail. By 8 hours, some of the parasites appeared necrotic.

Discussion

Many ultrastructural studies have revealed that different antimalarial drugs induce specific changes within plasmodial organelles and that typically the site of the earliest ultrastructural changes can be considered to be the initial site of action of these agents.[9-15] It can, however, be argued that these initial morphological changes could still represent secondary consequences of biochemical changes in other sites but this is probably less likely because previous data has always shown good correlation. Hence we performed an ultrastructural study to attempt to determine the site and mode of action of pyronaridine on plasmodial parasites.

Our findings indicated that this drug primarily causes alterations in the morphology of the parasitic food vacuoles. Previous studies have demonstrated that the erythrocytic forms of malarial parasites take up a portion of the host cytosol by endocytosis at cytostomes and form food vacuoles where host cell cytoplasm, which consists mostly of hemoglobin, is rapidly degraded.[16,17] The food vacuolar changes which include the formation of membrane-bound vesicles containing unaltered erythrocyte cytoplasm and decreased malarial pigment suggest that hemoglobin degeneration is impaired by pyronaridine. Various studies on the antimalarial effects of chloroquine have demonstrated initial damage to food vacuoles with alteration in the parasitic digestive system.[9,14,15,17] The morphological changes induced by pyronaridine may correlate with food vacuolar pH alterations similar to those induced by chloroquine.[18] Our findings suggest that pyronaridine possesses the same

target organelle as that of chloroquine i.e. the food vacuole. The comparison of the chemical structure of the two drugs reveals them to be similar, which may also support the suggestion (Fig. 8).

The results with P. berghei in vivo indicate that pyronaridine causes another early change in the parasites which was expressed ultrastructurally as the formation of multilamellate whorls in the pellicular complexes of trophozoites, schizonts and gametocytes. Interestingly, this pellicular complex effect persisted in the chloroquine-resistant strain of P. berghei treated with pyronaridine, in which the food vacuoles showed no significant change.[19] The action of this drug on both food vacuoles and pellicular complexes thus appears to be somewhat different from that of chloroquine and mefloquine in which the pellicular complexes are uninvolved.[14,20] This observation may provide a clue to explain the effectiveness of pyronaridine against chloroquine-and multidrug-resistant forms of malaria.

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Figure Legends

Figures 1-5. Electron micrographs of *P. berghei*.

Fig. 1: Thirty mins post-exposure. The trophozoite food vacuole (F) is enlarged and contains malarial pigment granules (P). Focal multilamellate whorls of the pellicular complex are present (arrow). X30,000.

Inset: Control. Small vesicles (f) contain individual pigment granules. X23,000.

Fig. 2: Two hrs post-exposure. Small vesicles (v) bounded by a single membrane and containing erythrocyte cytoplasm and membranous whorls (m). Fewer malarial pigment granules are seen in the large food vacuole (F). X17,000.

Inset: Multilamellate pellicular complex of a trophozoite (arrow). X33,000.

Fig. 3: Four hrs post-exposure. Schizont contains multilamellate whorls and swollen pellicular complexes (arrow). X33,000.

Fig. 4: Four hrs post-exposure. Multilamellate pellicular complex of a gametocyte (arrow). X29,000.

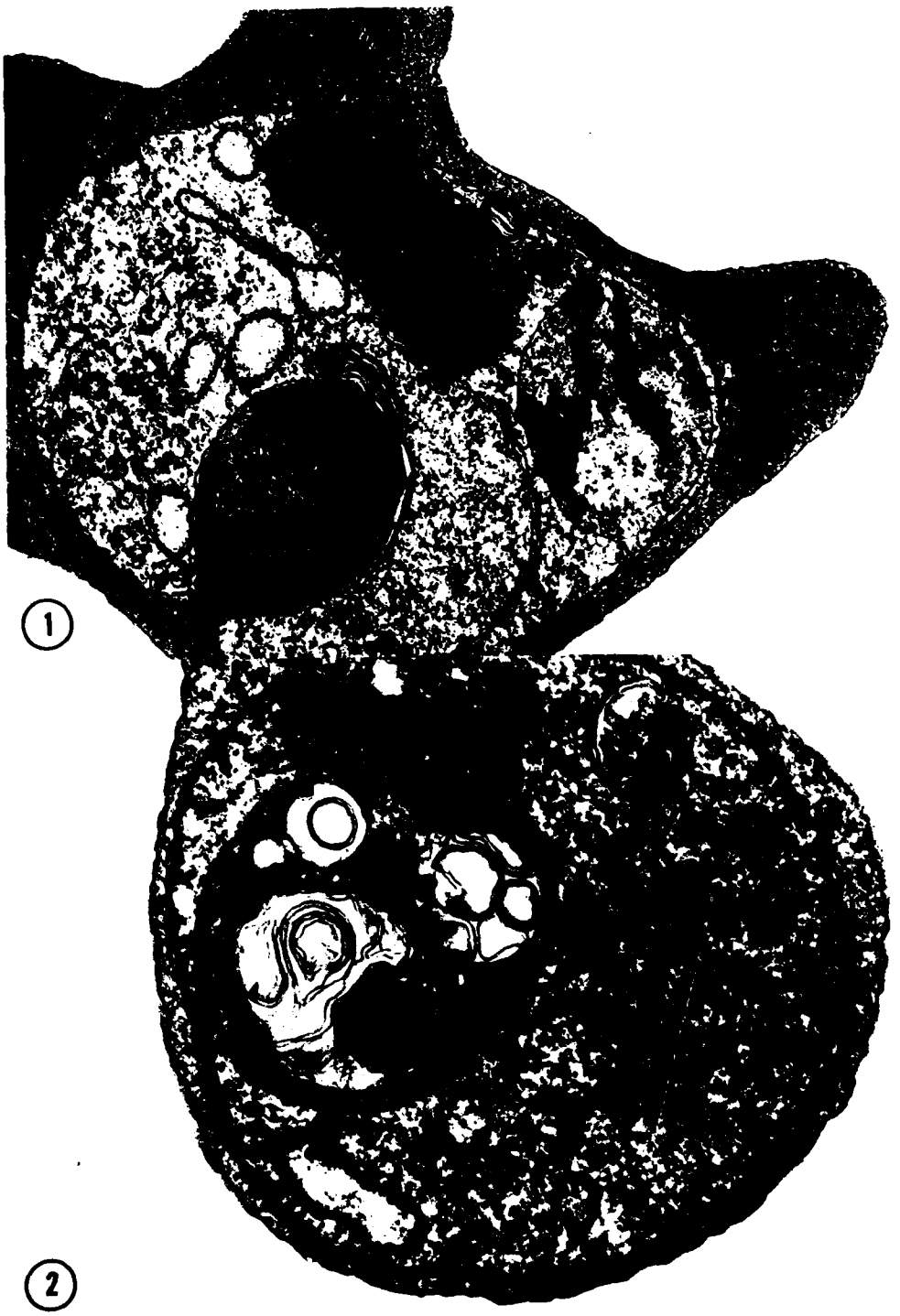
Fig. 5: Twenty-four hrs post-exposure. The parasite contains two large food vacuoles (F) that are filled with many small vesicles (v) limited by a single membrane. Malarial pigment granules are absent. X24,000.

Figures 6-7. Electron micrographs of P. falciparum.

Fig. 6: Two hrs post-exposure. The enlarged food vacuole (F) contains a small membrane-bound vesicle (v) containing host cell cytoplasm. X27,000.

Fig. 7: Four hrs post-exposure. The large food vacuole (F) contains a few smaller membrane-bound vesicles (v) and membrane whorls (m). The granular appearance of the contents of the small vesicles is identical to that of the host cell cytoplasm. X21,000.

Fig. 8: Chemical structure of pyronaridine.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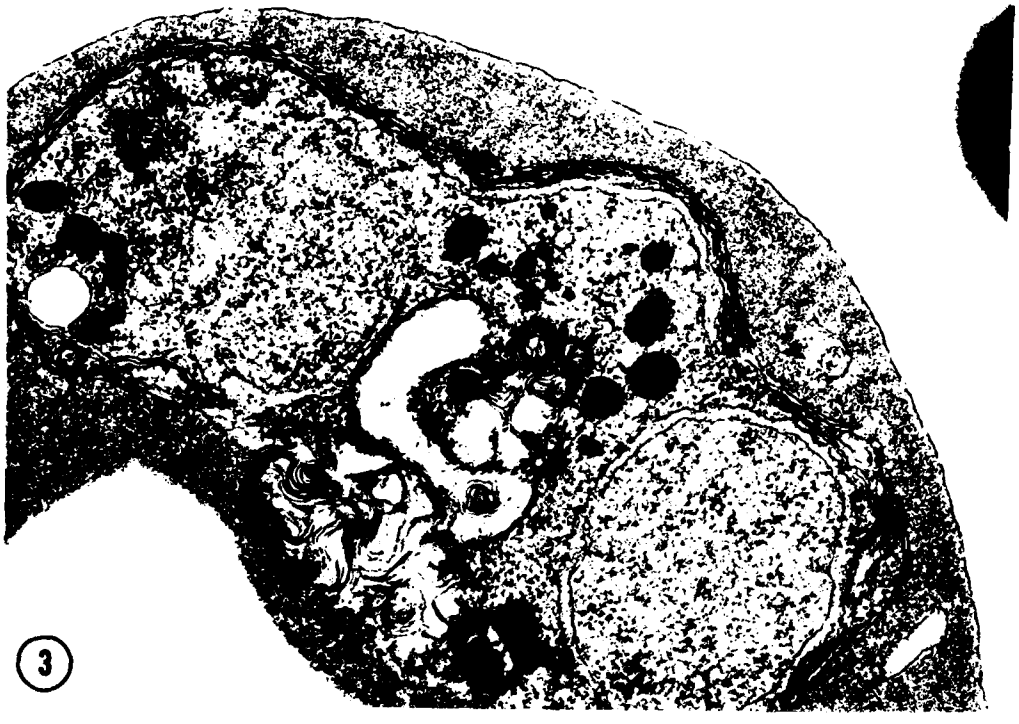

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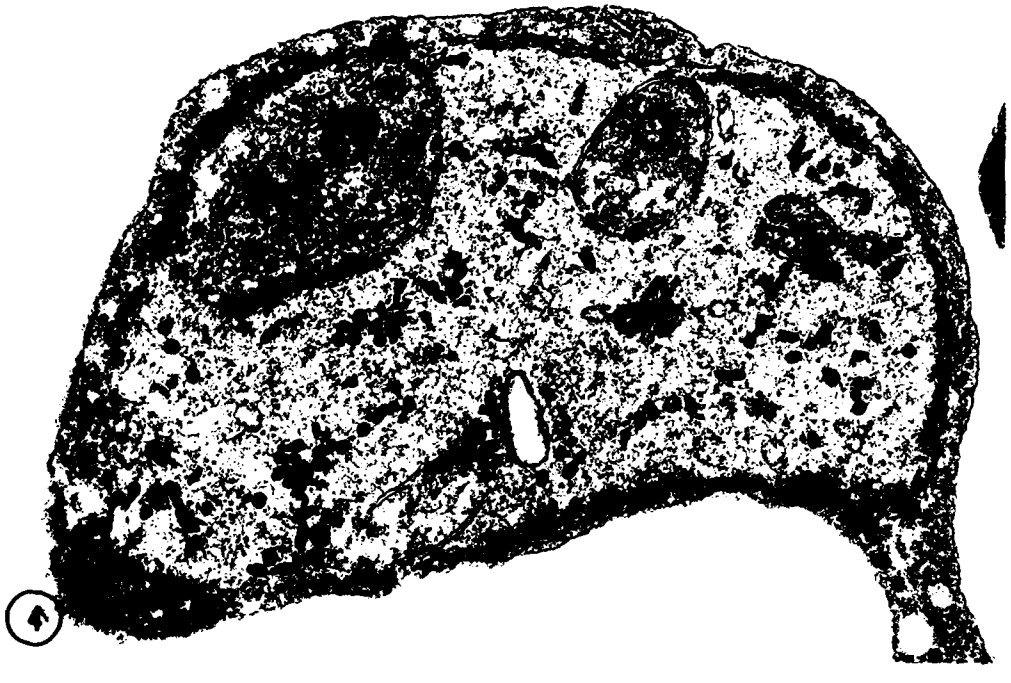

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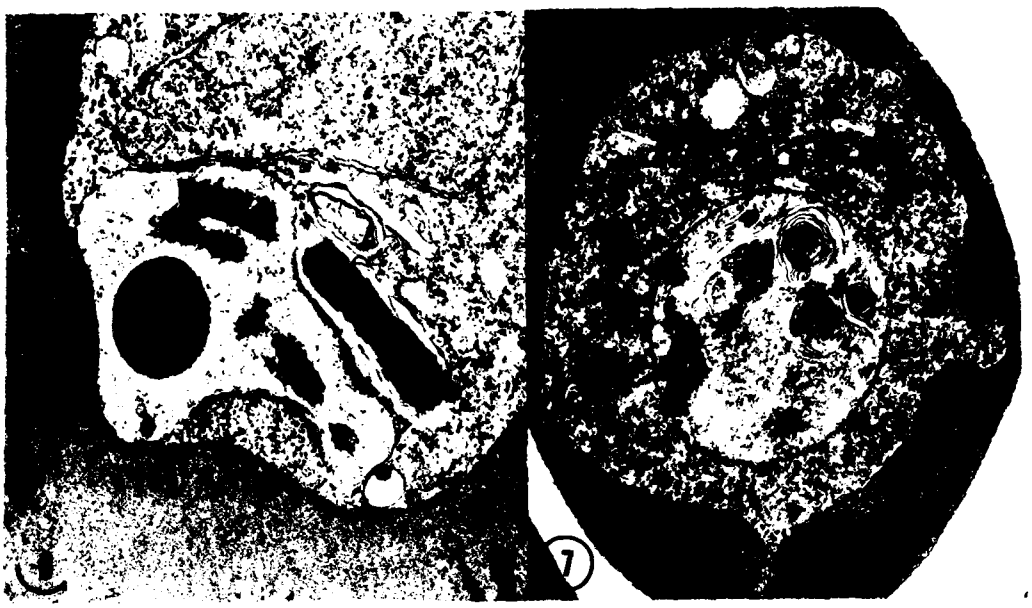

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