Effects of new antidermatophytic agent liranaftate on the ultrastructure of *Trichophyton mentagrophytes*

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The morphological effects of a new antifungal agent, liranaftate (M-732), on the ultrastructure of growing hyphae of the *Trichophyton mentagrophytes* strain were studied by scanning and transmission electron microscopy. After a short 24-h period of exposure at as low a concentration as 0.001 μg/ml, hyphal growth was markedly inhibited by the agent. Various cellular changes appeared, including irregular shape, bending, and shrinkage of the hyphae as well as swelling of the tip. Enlarged vacuoles finally occupied almost the entire inner part of the cells much earlier than in the non-treated cells. In the vacuoles, a myelin-like structure was observed. The vacuole membranes were damaged at several sites at a relatively high concentration of the agent. Numerous highly electron dense particles were detected in the cytoplasm, most of which were dispersed along with the inner stratum of the cell membranes. Lomasomes with many small vesicles were also seen at some sites between the membranes and the walls. At higher concentrations of the drug, an exfoliated outer layer of the walls, disrupted membranes, released cytoplasmic components, and concave hyphae were observed.

Key words: Liranaftate, Thiocarbamate antifungal, Electron microscopy, *Trichophyton mentagrophytes*

INTRODUCTION

Liranaftate (M-732), previously known piritetrate[1~4], is a potent antidermatophytic agent of thiocarbamate class. It was synthesized in 1982 by the Chemical Research Laboratory of Tosoh Corporation (formerly Toyo Soda Manufacturing Co., Ltd.), Shin-nanyo shi, Yamaguchi-ken. The first report on the *in vitro* and *in vivo* activities of this compound was made by Iwata *et al*[1,2], who compared it with the homologue tolnaftate[3]. Its mechanism of action was studied by Morita *et al*[4], who elucidated its selective inhibition of squalene epoxidase in *Cryptococcus neoformans* cells. This paper deals with scanning and transmission electron microscopy of the hyphae of a *Trichophyton mentagrophytes* strain exposed to varying concentrations of liranaftate for different periods of time.

MATERIALS AND METHODS

Antifungal. Liranaftate (M-732) was generously supplied through the Chemical Research Laboratory of Tosoh Corporation. The compound was dissolved in dimethyl sulfoxide (DMSO), and a stock solution of 20 mg/ml was kept at 4°C in the dark.

Organism. The IFM 40949 strain of *T. mentagrophytes* was used throughout this study.

Media. Sabouraud dextrose (2%) agar (SDA) and Sabouraud dextrose (2%) broth (SDB), both of which were supplemented with 0.1% yeast extract, were used.

Preparation of the cultures. The cultures, which had been incubated for a week at 26°C on SDA slants, were inoculated into a series of slide-culture technique using SDB of concentrations of 0, 0.001, 0.01, 0.1 or 1.0 μg/ml of liranaftate, and then incubated under the same culture conditons. Each of the cultures obtained was studied by electron microscopy.

Scanning electron microscopy (SEM). Hyphal cells were fixed with 2.5% glutaraldehyde (0.05 M phosphate buffer containing saline, PBS; pH 7.4) at 4°C for 2 h. After being washed with PBS, they were postfixed with 2.0% OsO_4 (0.1 M cacodylate buffer, pH 7.2) for 2 to 2.5 h. The fixed cells were dehydrated with a series of alcohol, substituted with amylacetate, and dried by the critical point drying method using liquid CO_2. Each of the dried specimens was coated with gold-palladium (60: 40) by ion sputtering and observed with a SEM, (Hitachi S-800) at 12 kV, as described[5].

Transmission electron microscopy (TEM). Hyphal cells fixed with glutaraldehyde were postfixed with 1.5% $KMnO_4$ at 4°C for 16 h. The cells grown at 26°C for 7 days were fixed with 1.5% $KMnO_4$ at 4°C for 16 h and additionally fixed with 2 % $KMnO_4$ at room temperature for 2 h. They were washed with distilled water, dehydrated in an acetone series (30, 50, 70, 80, 90 and 95% for 15 min each, three times in 100% aceton for 20 min each), and embedded in Quetol 812. Ultrathin sections prepared with a REICHERT OmU_3 ultramicrotome were stained with 6% uranyl acetate for 20 min and 0.5% lead citrate for 7 min. The samples thus obtained were observed with a TEM (JEM-1200 EXS) at 120 kV, as mentioned previously[6].

RESULTS

1. SEM observation

In the control culture grown without liranaftate, almost all the hyphae elongated normally, forming a large mass (Fig. 1). The growing hyphae, whose width averaged approximately 1.6 μm, had smooth surfaces. Those grown for 7 days elongated further and became densely entangled with each other. Their surfaces remained smooth.

The growth of the culture exposed to 0.001 μg/ml of liranaftate for 24 h was remarkably inhibited (Fig. 2). Part of the hyphal cells showed fairly remarkable changes: bending (←), remarkable shrinking (←—), and particles (◄) adhering to their surface.

In the culture exposed to 0.01 μg/ml of liranaftate for 24 h, the growth of the hyphae was also conspicuously inhibited (Fig. 3); some of the hyphal cells varied in width. Some of them were crushed (←—), and extruded branched hyphae. The length of the hyphal cells appeared shorter (←) than those grown in a drug-free medium, and the tips were blunt and swollen (◄).

The culture exposed to 0.1 μg/ml of liranaftate for 24 h showed partial disintegration of the cell walls of the hyphae (Fig. 4). The hyphal cells shrank (←—), curved (←), and were conspicuously twisted. Indefinite particles seemingly released from the hyphal cells were visible outside the cells (◄).

Under 1.0 μg/ml of liranaftate, the outer layer of the walls of some hyphal cells disintegrated and became entangled in the surface (Fig. 5 a). Some of the cell walls showed irregular shapes and sizes, taking on a curved (←) or sheet-like appearance (←—) with attached fibrillar substances. The hyphal tips were swollen (◄). The hyphal cells differed considerably in width, and were smashed and flattened (Fig. 5 b). Small particles appeared to cling to the hyphal surface. The outer layer of the cell walls exfoliated with resultant disclosure of a rough and granular inner face, and fibrillar materials were observed clinging to the concave layers (Fig. 5 c). Particles seemingly detached from the hyphal cells adhered to the surface.

The growth of the hyphae treated with 0.001 μg/ml of liranaftate for 7 days was inhibited to some extent (Fig. 6). The hyphal cells were swollen at their tips. Some of the cells were bent (←), and many of them were shrunken or flattened (←—).

The hyphae exposed to 0.01 μg/ml of liranaftate for 7 days were more markedly inhibited, showing levels similar to those exposed to 1.0 μg/ml of the drug for 24 h.

The strongest effects of the drug on hyphal growth and morphology were recognized under exposure to 1.0 μg/ml of liranaftate for 7 days: most

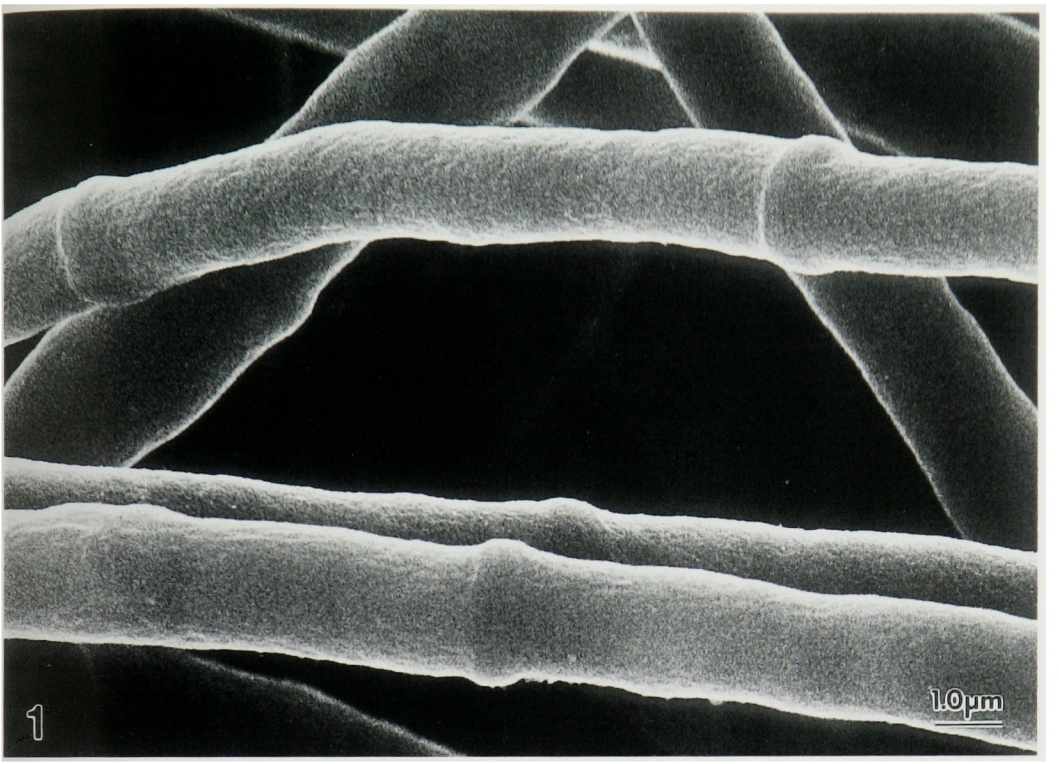


Fig. 1. SEM image of untreated control hyphae grown for 24 hours. ×9,000

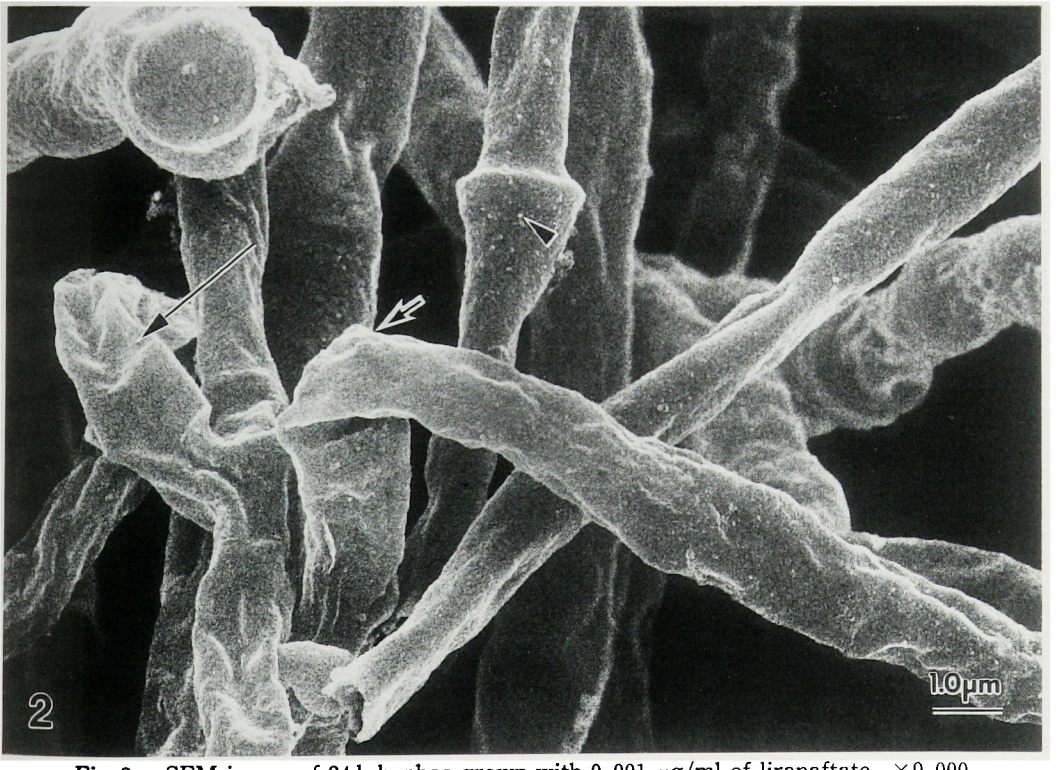


Fig. 2. SEM image of 24 h hyphae grown with 0.001 μg/ml of liranaftate. ×9,000

Fig. 3. SEM image of 24 h hyphae grown with 0.01 μg/ml of liranaftate. ×4,500

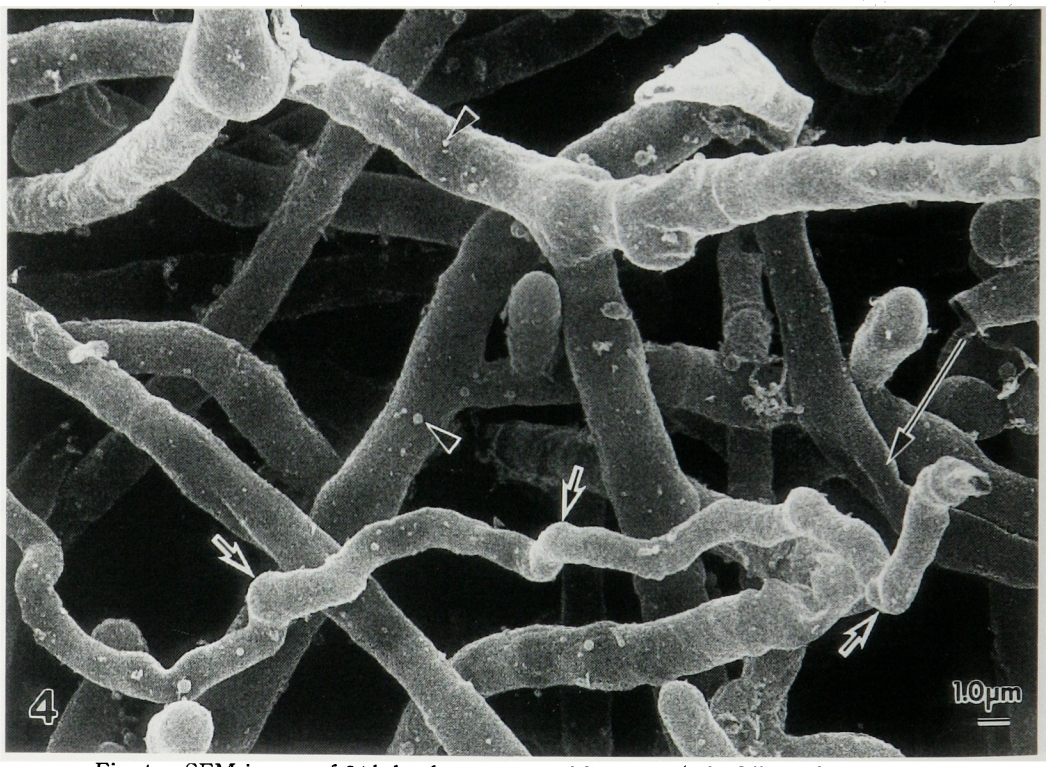


Fig. 4. SEM image of 24 h hyphae grown with 0.1 μg/ml of liranaftate. ×4,500

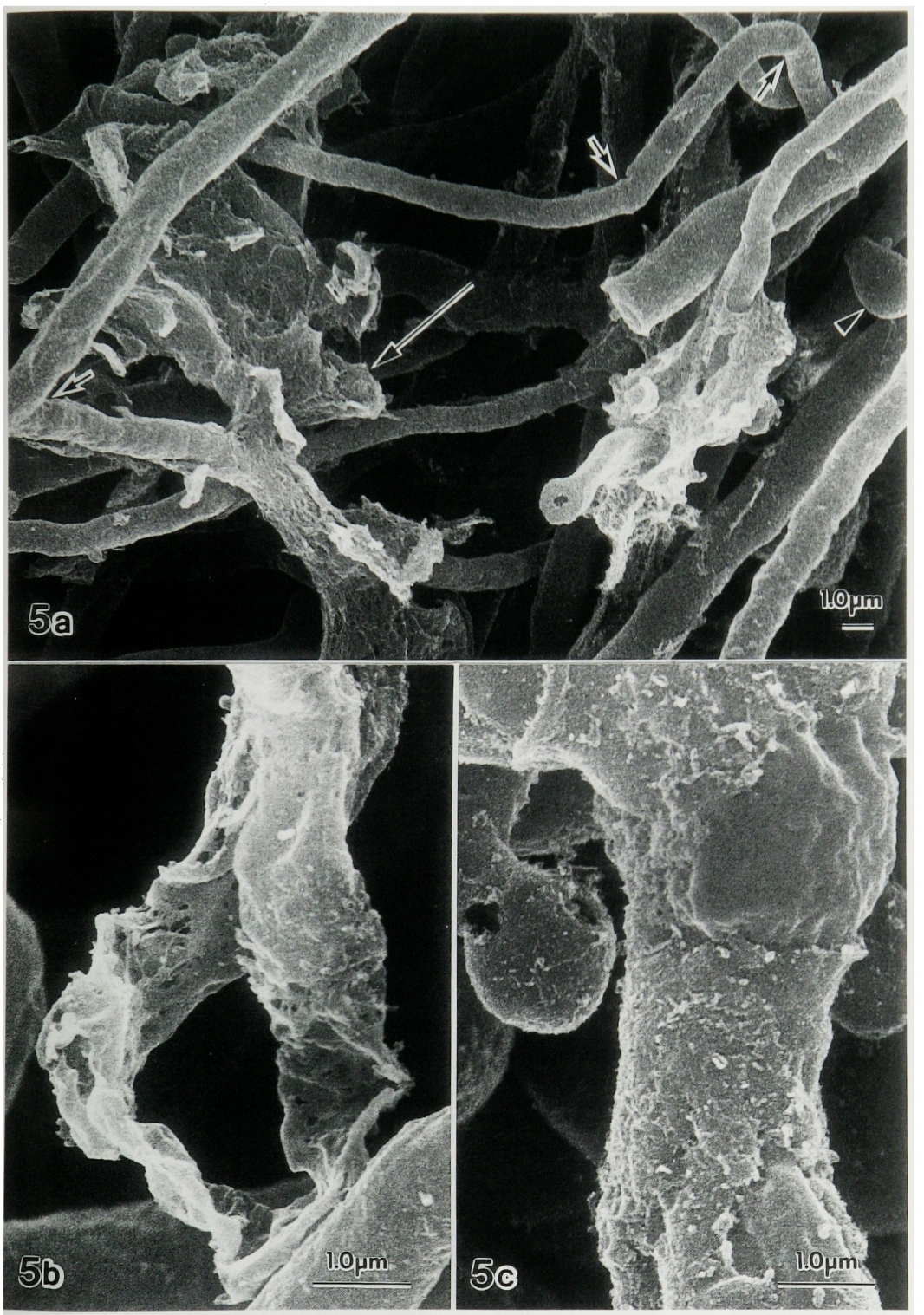


Fig. 5. SEM images of 24 h hyphae grown with 1.0 μg/ml of liranaftate. a, ×4,500; b and c, ×15,000

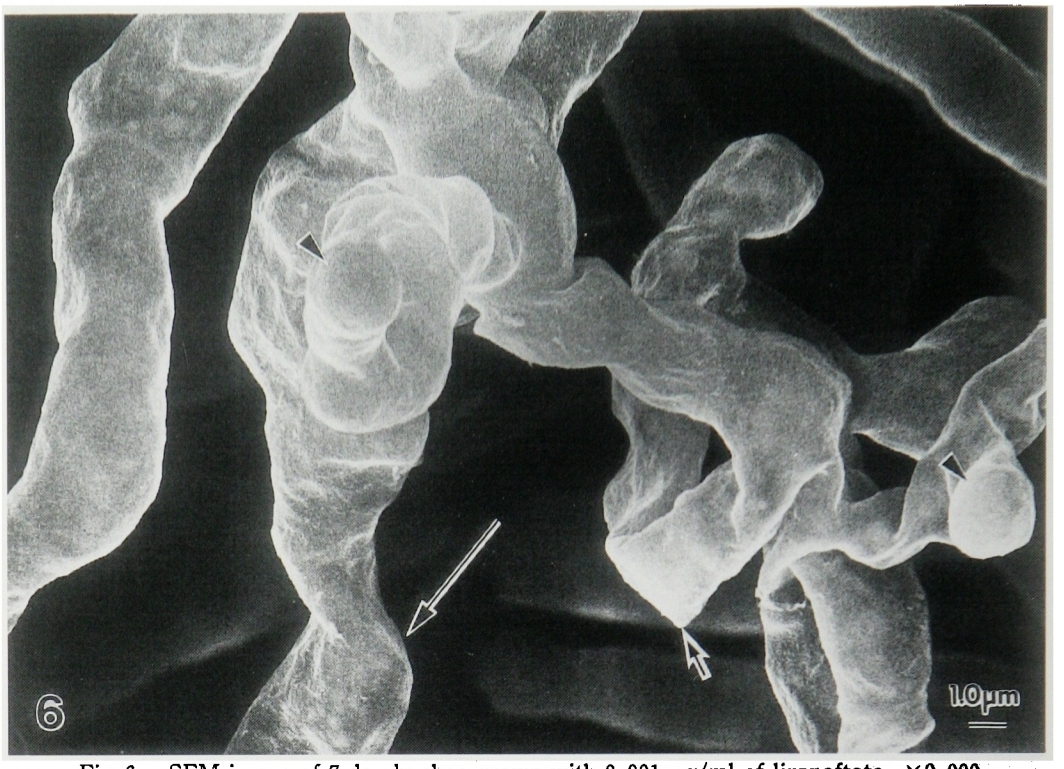


Fig. 6. SEM image of 7 day hyphae grown with 0.001 μg/ml of liranaftate. ×9,000

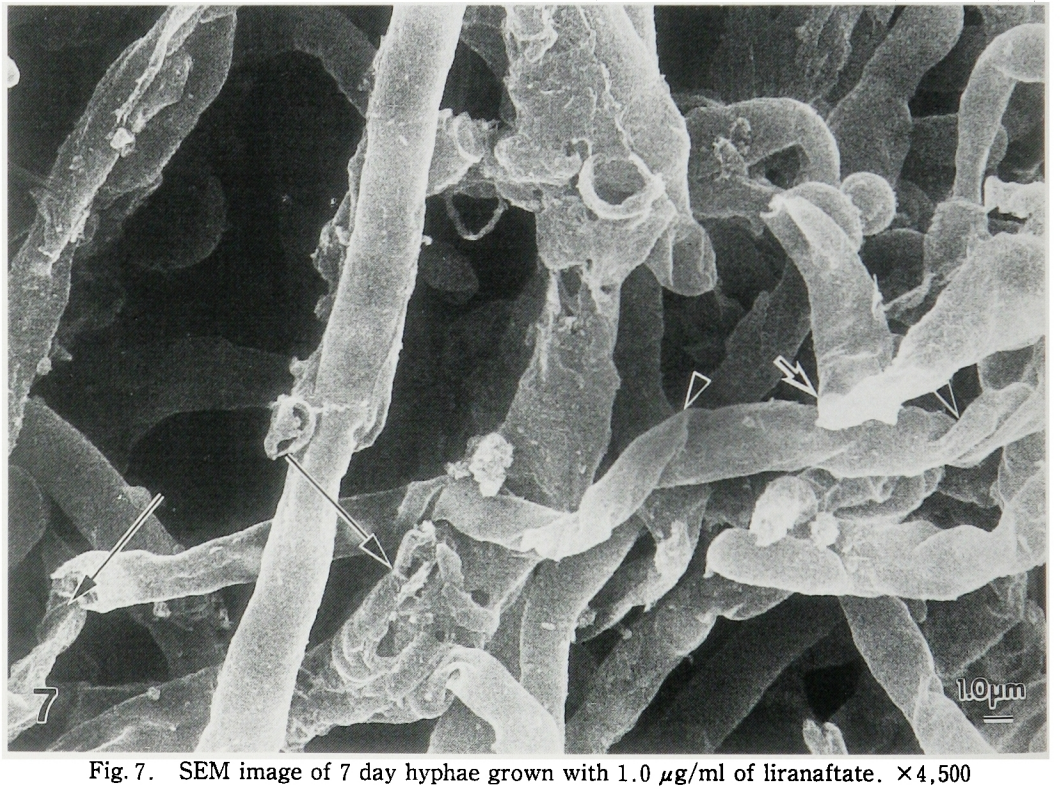
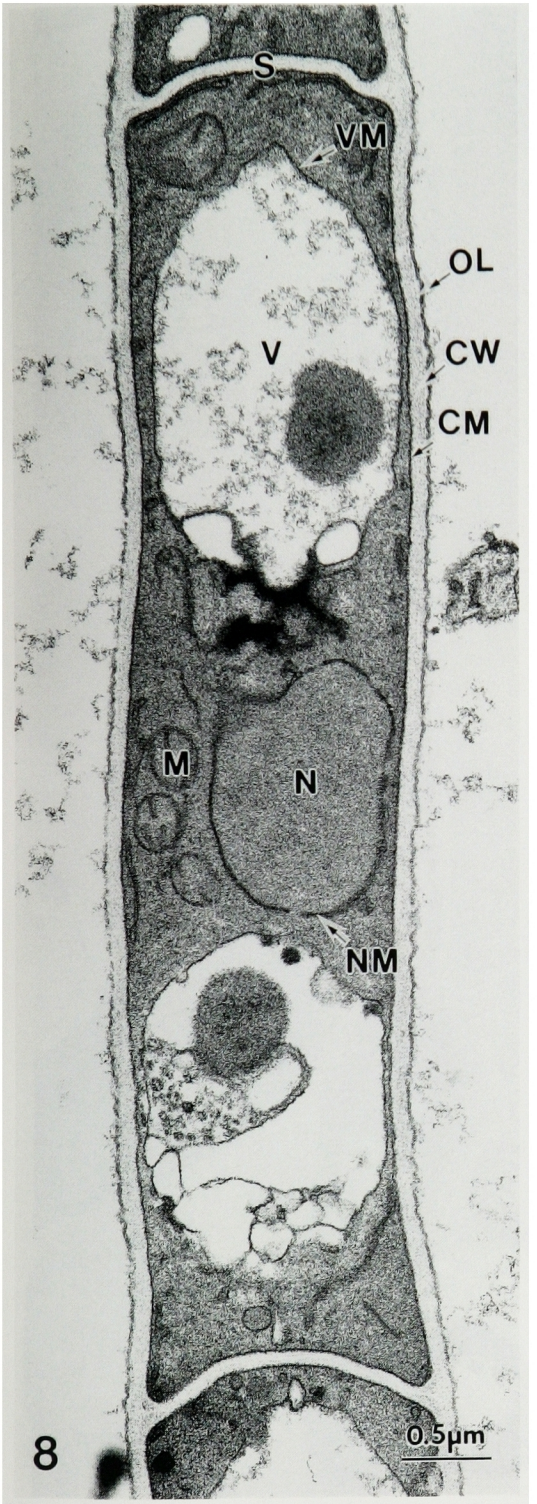
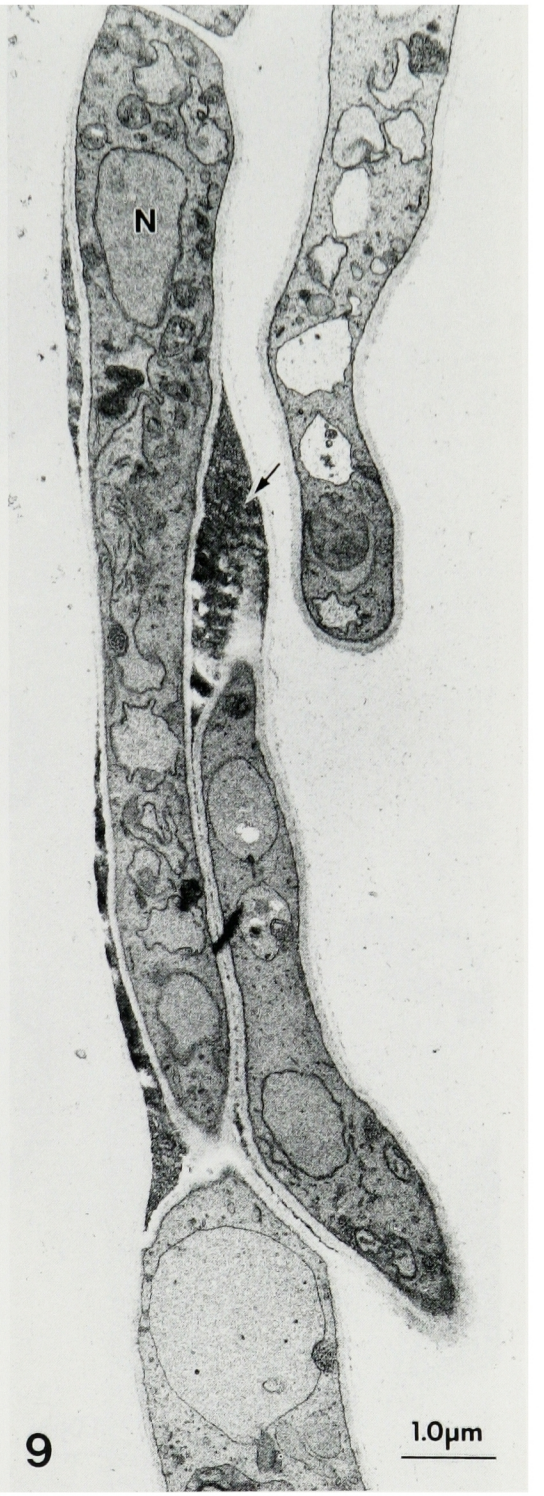


Fig. 7. SEM image of 7 day hyphae grown with 1.0 μg/ml of liranaftate. ×4,500



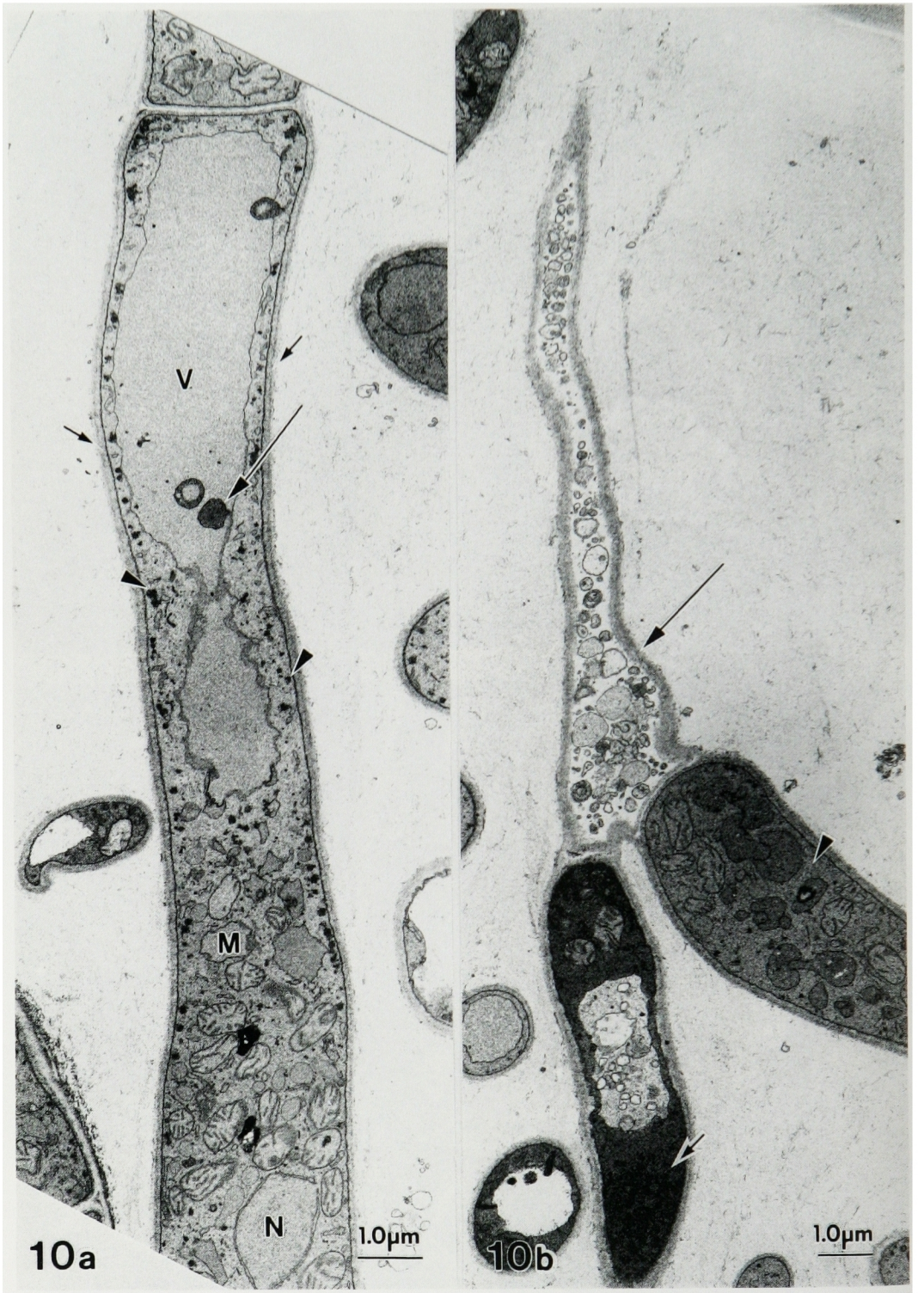
CM, cell membrane; CW, cell wall; M. mitochondrion; N. nucleus; NM, nuclear membrane; OL, outer layer of cell wall.

Fig. 8. TEM image of an untreated control hypha grown for 24 hours. ×24,000



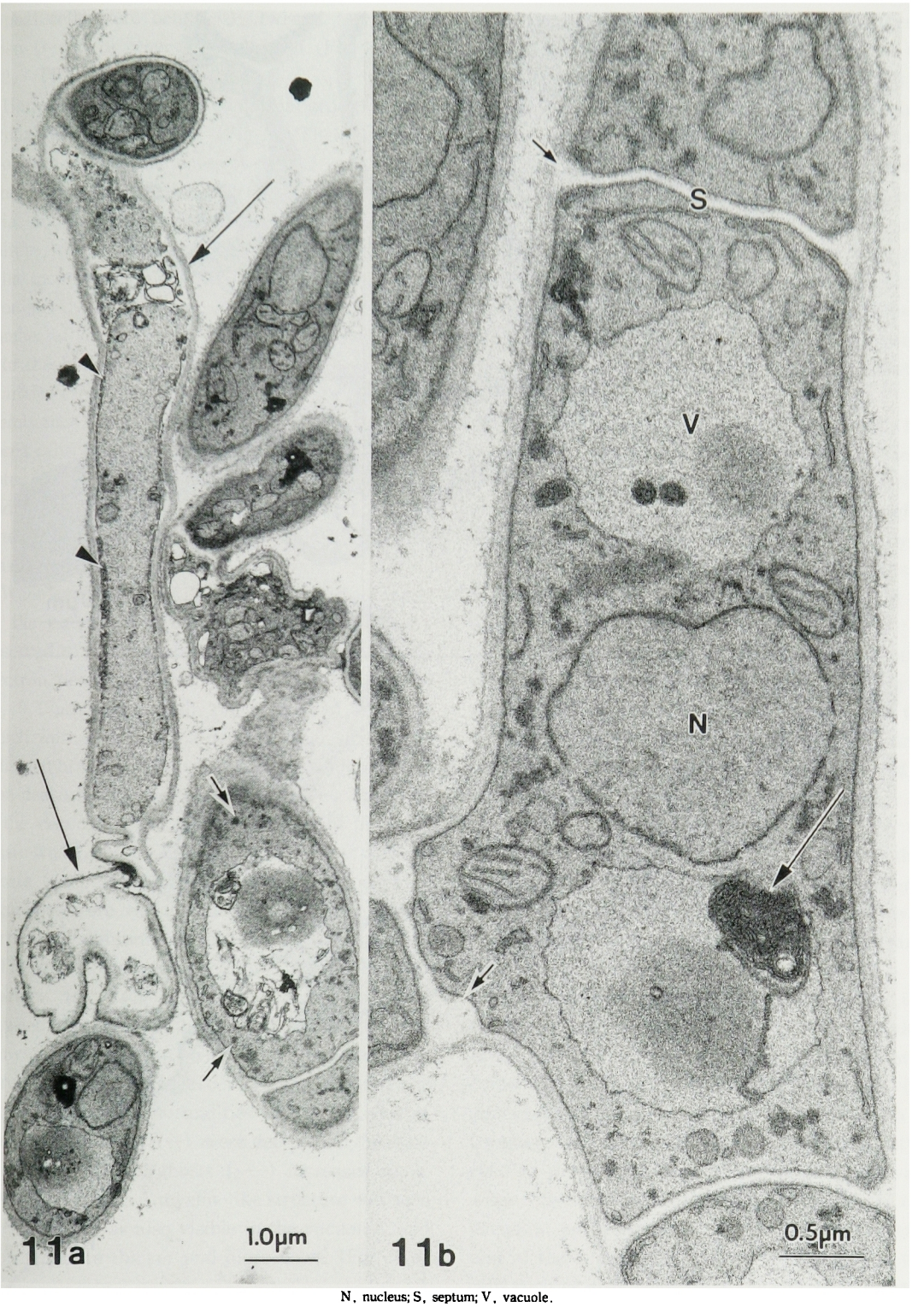
N, nucleus.

Fig. 9. TEM image of 24 h hyphal cells grown with 0.001 μg/ml of liranaftate. ×12,000



M, mitochondrion; N, nucleus; V, vacuole.

Fig. 10. TEM images of 24 h hyphal cells grown with 0.01 μg/ml of liranaftate. ×10,000



N, nucleus; S, septum; V, vacuole.

Fig. 11. TEM images of 24 h hyphal cells grown with 0.1 μg/ml of liranaftate. a, ×11,000; b, ×26,000

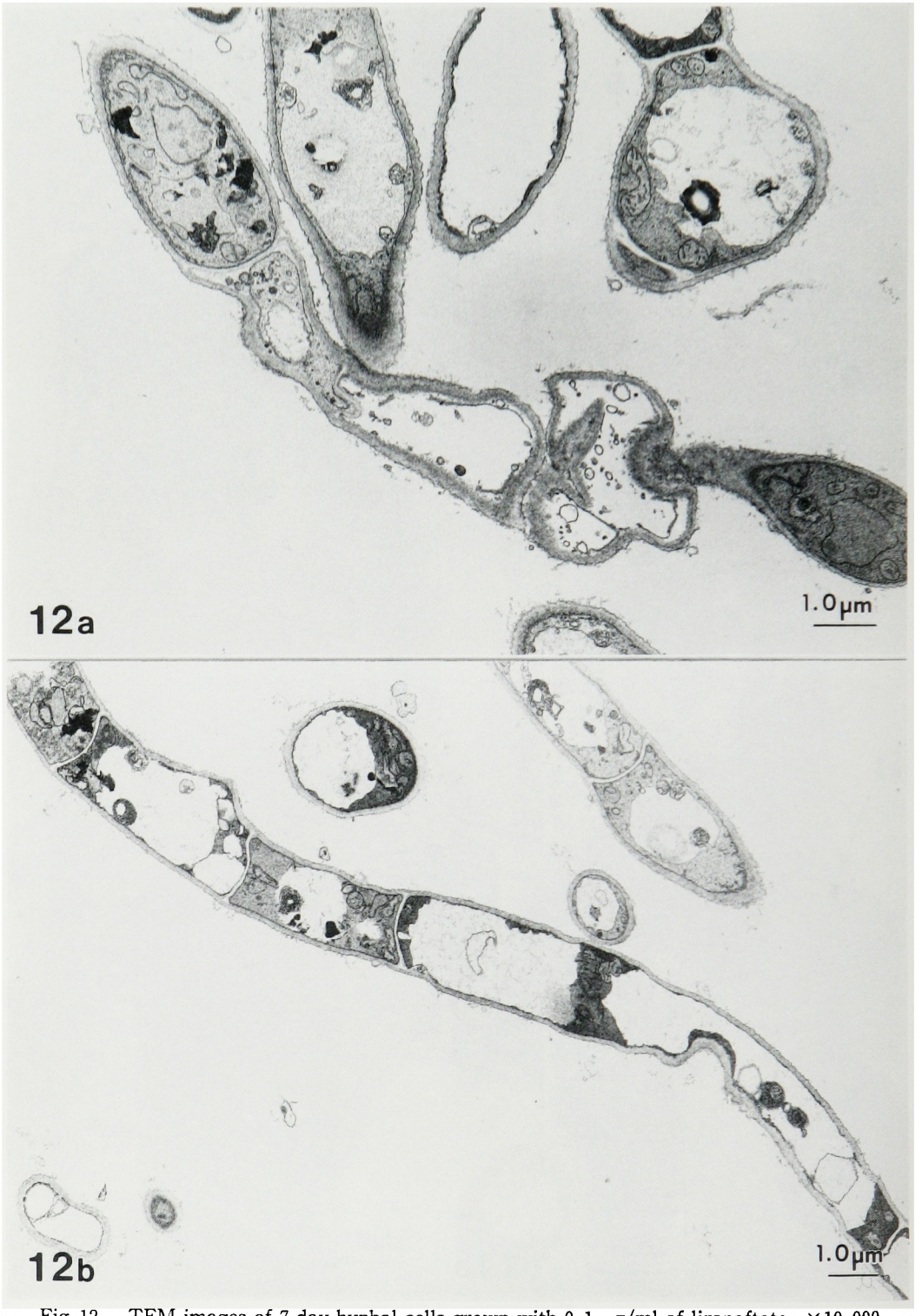


Fig. 12. TEM images of 7 day hyphal cells grown with 0.1 μg/ml of liranaftate. ×10,000

hyphal cells were bent (←), twisted (➡), shrunken (⟵), or otherwise deformed (Fig. 7).

2. TEM observation

Untreated cultures showed normal growth; all the hyphal cells grew straight (Fig. 8). Their cell walls showed a uniform thickness of ca. 0.1 μm, and the inner cellular organelles such as nuclei, mitochondria, and vacuoles appeared regular. In the 7 day-culture, the hyphae also grew straight; the hyphal cells increased in length and width much more prominently than in the 24 h-culture. A greater number of vacuoles was seen in some cells.

In the hyphal cells exposed to 0.001 μg/ml of liranaftate for 24 h, the electron density of the cell membranes was somewhat higher than in the unexposed cells (Fig. 9). Although no abnormality was noted in the inner cellular organelles such as nucleus and mitochondria, some of the hyphal cells bent or deteriorated (←), and the width of the hyphal cells was no longer uniform.

When exposed to 0.01 μg/ml of liranaftate for 24 h, the outer layers of the cell walls (←) were observed to be exfoliated (Fig. 10 a). Many highly electron dense particles (➡) with a diameter of ca. 0.1 μm appeared in the cytoplasm, most of which were distributed along the inner side of the cell membrane. Some vesicles with a highly electron dense membranous structure (⟵) appeared in the vacuoles. The electron density of the cytoplasm was lower (➡) and/or higher (←), and cell ghosts (⟵) were seen (Fig. 10 b). Various inner organelles were variable in size and shape, being particularly different between the branched hyphae.

The hyphal cells exposed to 0.1 μg/ml of liranaftate for 24 h displayed lacuna structures between the cell membrane and cell walls, and many small vesicles, so-called lomasomes (➡), were observed in those structures in addition to the above-mentioned abnormalities (Fig. 11 a). Numerous small particles (←) were detected in the cytoplasm, and the cell ghosts (⟵) increased remarkably in number. A myelin-like structure was seen (⟵) which was also visible in the vacuoles, and the septa were thicker and more wavy (Fig. 11 b, ←).

Exposure to 1.0 μg/ml of the drug caused partial lysis of most of the hyphal cells and severe deterioration of most of the organelles, leading to the formation of vesicles, vacuoles, or membrane fragments. The vacuoles enlarged greatly and their membranes were partially damaged. Most of the cells bent and markedly shrank (photos omitted).

In the hyphal cells exposed to 0.001 μg/ml for 7 days, lacunar structures appeared between the cell wall and membrane, and many vesicles with grooves or a flat shape filled the lacuna. Some vesicles were present in the cell walls, and the cell membranes were partially broken or had disappeared. The vacuoles were markedly enlarged (photos omitted).

Under exposure to 0.01 μg/ml of liranaftate, the vacuoles conspicuously enlarged, and their membranes were profoundly damaged; huge myelin-like structures were visible in the vacuoles. Many highly electron dense particles were also seen in the cytoplasm; most of them were located close to the cell membrane, and a few others were observed in an aggregated state (photos omitted).

Exposure to 0.1 μg/ml of liranaftate induced protound deterioration of the hyphal cells; the cytoplasm almost completely disappeared in most of the cells, and the inner cellular organelles were disrupted or lost. A myelin-like structure was observed in the vacuoles (Fig. 12).

DISCUSSION

Through SEM and TEM we demonstrated that the antifungal agent liranaftate (M-732) profoundly affected the hyphal cells of a *T. mentagrophytes* strain (IMF 40949) even at the very low concentration of 0.001 μg/ml after exposure for 24 h or longer. The ultrastructural changes caused by the compound can be summarized by the following three features: (1) alterations in hyphal growth evidenced by waving, bending, and twisting, (2) abnormalities in hyphal shape and structure such as irregular forms, and cell-derived components attached to the cell surface, enlargement of the vacuoles, appearance of a myelin-like structure in the vacuoles, particles in the cytoplasm, and degeneration of the cytoplasm, and (3) breakdown of the cells such as disintegration of the cell walls and the release of cytoplasmic components. Features (1)

and (2) were observed under diluted concentrations of the compound, feature (2) differed according to the concentration used, and feature (3) was observed at higher concentrations. These features were found to depend on the incubation and/or exposure time, with a longer periods of exposure having a severely damaging effects on the hyphal cells.

It is to be noted that liranaftate caused various severe degenerative or necrotic morphological changes. This suggests a possible effect(s) of the compound on the formation of the cell walls. These profound morphological changes are considered to reflect the very potent *in vitro* and *in vivo* antidermatophytic activity of liranaftate reported by Iwata *et al*[1,2]

Borgers and van de Ven[1] showed structural deterioration in *Trichophyton rubrum* after treatment with itraconazole, a triazole antifungal, using transmission and scanning electron microscopy. They demonstrated the initial accumulation of vesicular materials in the walls and internal vacuoles of the growing tips of the hyphae; primary alterations at the cell periphery were seen only in growing cells. These findings agreed, to some extent, with the changes in the surface structure profiles of cells treated with liranaftate. Such similar cell surface-damaging effects have also been observed with the hyphae of *T. mentagrophytes* when exposed to itraconazole[8] and tolciclate[9], a thiocarbamate antifungal, both of which are ergosterol inhibitors.

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新規抗皮膚糸状菌剤リラナフタートの *Trichophyton mentagrophytes* の微細構造におよぼす影響

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走査型および透過型電子顕微鏡を用いて，抗真菌剤リラナフタート（M-732）の白癬菌 *Trichophyton mentagrophytes* に対する形態的影響について検討した。この薬剤によって，菌糸の成長が阻害され，菌糸の枝分かれ，形態異常，屈曲，収縮，先端部の膨潤が観察された。液胞は巨大化して細胞の大部分を占めるようになった。このような液胞内にはミエリン状の構造体が出現し，液胞膜は薬剤濃度の高い状態ではところどころで破れた。細胞質内に電子密度の高い顆粒が多数出現し，その多くが細胞膜の内側に沿って分布していた。細胞壁が細胞膜から剝離し，その間隙に多くのロマソームが出現した。薬剤の影響が増大するにつれて，細胞壁の最外層が剝離し，細胞膜も破壊され，細胞内物質が流出し，それらが集合してシート状の塊を形成した。最終的には，ほとんどの菌糸細胞が空洞化した。リラナフタートの *T. mentagrophytes* 菌糸への阻害影響は薬剤濃度 0.001 μg/ml の低い薬剤濃度で 24 時間培養しても現れた。薬剤の阻害影響は濃度が高いほど，培養時間が長いほど大きく現れた。

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