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ULTRASTRUCTURAL ALTERATIONS INDUCED BY AMPHOTERICIN B IN THE PLASMA MEMBRANE OF *EPIDERMOPHYTON FLOCCOSUM* AS REVEALED BY FREEZE-ETCH ELECTRON MICROSCOPY

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SUMMARY

Freeze-etch electron microscopic observations demonstrated that Amphotericin B, a potential antifungal polyene induced profound ultrastructural alterations in the plasma membrane of cells of a human pathogenic fungus, *Epidermophyton floccosum*; aggregation of membrane-associated particles (85-Å) and formation of depressions (or craters) on the inner fracture face.

Thin-sectioning electron microscopy showed "vesiculation" of plasma membranes of Amphotericin B-treated *Epidemophyton* cells.

It can be suggested from the preliminary results that the fungal cells which contain ergosterol as the major sterol are highly sensitive to Amphotericin B.

INTRODUCTION

The polyene antibiotics which are characterized by the possession of a macrolide ring of carbon atoms have been known to be potential antifungal agents and been widely used for clinical treatment of systemic fungal diseases. However, the mode of action of polyene antibiotics is not yet completely understood. Since most polyene antibiotics are known to be highly effective against fungi as well as algae, protozoa and mammalian cells which contain sterols, but are insensitive against bacteria devoid of any sterol, it would appear that these antibiotics require the presence of sterols as a prerequisite for their antifungal activity [1–5]. In fact, several lines of evidence obtained from studies with artificial [6–10] and biological [2, 11–14] membranes have shown that the polyene antibiotic binds to sterol molecules in membranes, causing leakage of essential cellular constituents and thereby impairing functions of biological membranes. In addition to these biochemical studies, direct evidence for binding between polyenes and sterols in the membrane was provided by fluorimetric [15–17] and spectrophotometric studies [16, 18].

On the other hand, the membrane lesions which are produced by polyene–sterol interactions have been examined with negative staining electron microscopy, demonstrating the formation of "pits" [19, 20]. Recently, the freeze-fracture technique

proved to be a powerful tool for observing ultrastructure within the plane of the membrane, and by the use of this technique some interesting findings have been presented indicating the profound effects upon the ultrastructure of *Acholeplasma* [21, 22] and erythrocyte [20] membranes containing cholesterol. However, to our knowledge there is little information concerning the ultrastructural alterations induced by antifungal polyenes in the ergosterol-containing membranes of fungal cells. Therefore, it will be interesting to investigate by freeze-etching and thin-sectioning techniques the effects of Amphotericin B, known to have a profound activity against fungal cells, on the ultrastructure of the plasma membrane of a human pathogenic fungus, *Epidermophyton floccosum*.

MATERIALS AND METHODS

Growth of organism

E. floccosum TEF-30 was grown for 5 days at 28 °C in shake flasks containing Sabouraud's medium (4 % glucose, 1 % Polypeptone, and 0.5 % yeast extract).

Incubation of E. floccosum cells with Amphotericin B

The harvested cells were incubated for 1 h at 28 °C with Amphotericin B (Fungizone, E. R. Squibb and Sons, Inc., New York) at a final concentration 2 μg/ml. As the control cells, *E. floccosum* cells were incubated with sodium deoxycholate at the same concentration as that in Fungizone.

Freeze-etching electron microscopy

Samples were prefixed for 5 h at 0 °C with 2 % glutaraldehyde buffered with 0.1 M cacodylate buffer, pH 7.4, and transferred to 40 % glycerol in 0.85 % NaCl. The specimens, on a brass holder, were rapidly frozen in Freon 12 at liquid N_2 temperature. Freeze-fracturing and etching were carried out using a Hitachi HFZ-1 freeze-etching device. Replication was performed with platinum-carbon and the replicas were coated with carbon. After floating on distilled water, the replicas were cleaned in a hypochlorite solution, followed by soaking in 75 % H_2SO_4 for 24 h. After this cleaning procedure, the replicas were given three rinses in water prior to collection on 400-mesh grids. The electron microscopic observation was performed with a Hitachi HS-8 electron microscope.

Thin-sectioning electron microscopy

Thin-sectioning electron microscopy was performed according to the procedure described previously [23].

Lipid extraction and gas–liquid chromatography

The freeze-dried mycelia were extracted with chloroform–methanol (2 : 1, v/v) for 18 h and then refluxed for 2 h. The combined extract was concentrated. For analysis by gas–liquid chromatography, an aliquot of total lipids was hydrolysed in 2 M KOH in 50 % ethanol for 2 h at 100 °C. The benzene extract was analysed isothermally at 271 °C in a JEOL Model JGC-1100 gas chromatograph. The conditions for operation are the same as described previously [24].

RESULTS AND DISCUSSION

The ultrastructural appearance of freeze-etched plasma membrane of the control cells of *E. floccosum* is shown in Fig. 1a and b demonstrating the marked differences between the inner fracture face and the outer fracture face. It is apparent that the inner fracture face shows a random distribution of a large number of 85-Å particles associated with membrane (Fig. 1a), whereas the outer fracture face appears smooth with only some randomly distributed particles (Fig. 1b). Although the chemical nature of these membrane-bound particles is thought to be protein (or glycoprotein) [25, 26], the function of the particles remains still unknown.

Incubation of *Epidermophyton* cells with a heptane macrolide antibiotic Amphotericin B (2 μg/ml) induced profound ultrastructural alterations on the inner fracture face, decrease in density of membrane-bound particles, formation of depressions (or craters) with varying depths and aggregation of particles accompanied with smooth plaques devoid of particles (Fig. 2a). In contrast, such predominant aggregation of particles was not significantly observed on the outer fracture face but similar depressions were visible (Fig. 2b). It can be suggested that the aggregation of particles might be due to translational movement of the particles within the plane of the membrane which was caused by rearrangement of lipids through ergosterol–Amphotericin interaction. There would be two possible explanations for the mechanism of this sterol–polyene interaction causing aggregation of the membrane intercalated particles; first, removal of ergosterol molecules from the membrane as a sterol–polyene complex and second, "inactivation" of ergosterol by complexing with the polyene in the membrane but no leakage of the sterol. The latter might be plausible since we were not able to find any significant difference in the ergosterol content in the fungal cells as determined by gas-chromatographic analysis. Hence, it can be concluded that Amphotericin B interacts with ergosterol so that the sterol is withdrawn from its interaction with membrane phospholipids, leading to loss of the key function as a membrane stabilizer regulating the membrane fluidity. Thus the altered fluidity, e.g. liquid crystalline–gel phase, causes aggregation of the membrane particles as observed with *Acholeplasma* [27]. It would be also possible that decrease in the number of particles suggests that protein particles were embedded deeper into the interior part of membrane following the lipid phase reorganization or removed from the hydrophobic region in the membrane.

No "pores" [19] could be visible as holes which transverse the membrane. The absence of "pores" on the fracture faces is comparable with the cases of *Acholeplasma* [21, 22] and erythrocyte [20, 21] membranes incubated with filipin which has been known to cause drastic alterations in membranes. Recently, Seeman et al. [28] demonstrated by freeze-etch electron microscopy of saponin-treated erythrocyte membranes that 40–50-Å-wide pits are uniformly distributed in the extracellular surface of the membrane, and suggested that the pits may be the place from which the cholesterol molecules were extracted. Also similar findings were shown in the immune-lysed erythrocyte [29]. Verkleij et al. [21] have pointed out that Amphotericin B (20 μg/ml) does not visibly affect the freeze-etch ultrastructure of erythrocyte, *A. laidlawii* cells and lipid dispersion, all of which contain only cholesterol. It is therefore of interest to note that our present study demonstrated that Amphotericin B at a lower concentration (2 μg/ml) induced the profound alterations on the fracture faces of

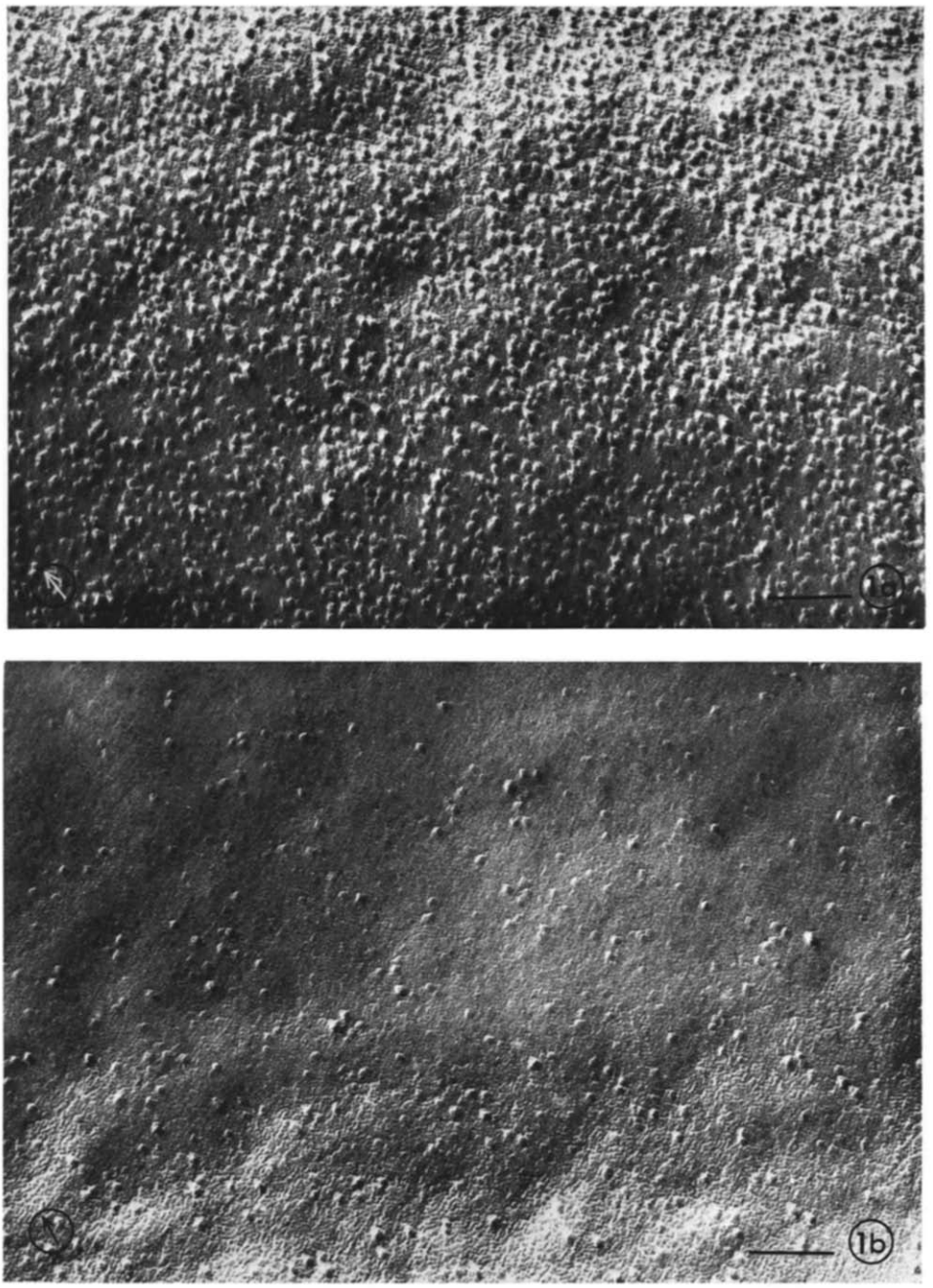

Fig. 1. Freeze-etched views of an inner fracture face (a), demonstrating a homogeneous random distribution of membrane-bound particles and an outer fracture face (b) of the intact plasma membrane of *E. floccosum* cell. More particles are visible on the inner fracture face than on the outer fracture face. The arrow and the scale indicate shadowing direction and length of 0.1 μm, respectively. Magnification, $\times$110 000.

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DISK TO SPHERE TRANSFORMATION OF ERYTHROCYTES, INDUCED BY 1-ANILINO-8-NAPTHTHALENESULFONATE

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SUMMARY

1. The agent 1-anilino-8-naphthalenesulfonate (ANS) has been found to induce a transformation of bovine erythrocyte from a biconcave disk to a smooth sphere through a crenated sphere.

2. The process of the transformation was observed with a scanning electron microscope. With the increase of ANS concentration crenations became steeper and more numerous at first, and the erythrocyte finally became spherical with a smooth surface.

3. The transformed erythrocyte was returned reversibly to the original shape by removing ANS as far as observed under an optical microscope.

4. Hematocrit measurements showed that the volume of the erythrocyte did not change throughout the transformation. This indicates that the surface area decreased by 10 % at the final stage.

5. Coulter counter measurements suggest that crenations are not removed but flatten out in the process from crenated sphere to smooth sphere.

INTRODUCTION

Normal mammalian erythrocytes are known to have the shape of a biconcave disk. Their shape becomes a crenated disk, a crenated sphere, and a smooth sphere in succession, either by the addition of amphiphilic–anionic agents, by an increase in pH, or by lowering the ATP level in erythrocytes (crenated type transformation). On the other hand their shape becomes a shallow cup, a deep cup, and a smooth sphere in succession, either by the addition of amphiphilic–cationic agents or by a decrease in pH (cup type transformation) [1–3]. Since erythrocytes have not any subcellular structures, these morphological changes should be attributed to a structural change of the cellular membrane of erythrocyte.

It was found by the authors that a crenated type transformation is induced by

Abbreviation: ANS, 1-anilino-8-napthalenesulfonate.

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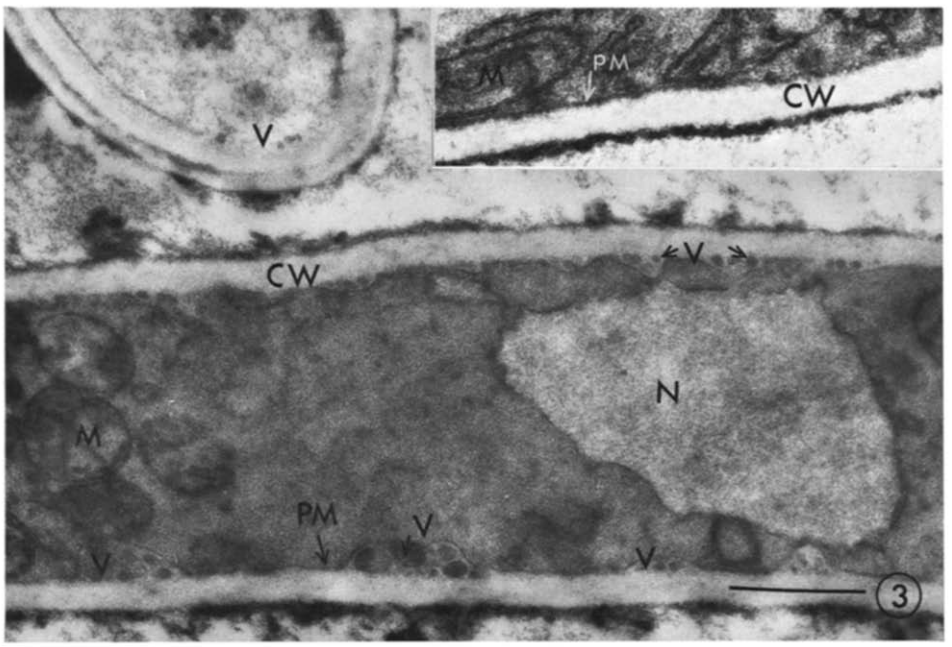

Fig. 3. Thin-sectioned ultrastructure of the Amphotericin B-treated cell of *E. floccosum*. The remarked "vesiculation" of the plasma membrane can be seen in many areas along the membrane but no significant alteration is visible in other membrane structures. The inset shows the plasma membrane of the control cell. N, nucleus; V, "vesicles"; M, mitochondria; PM, plasma membrane; CW, cell wall. The scale indicates 0.5 μm. Magnification, $\times$ 34 200.

Epidermophyton plasma membrane. Since the total lipid of fungal whole cells was found to contain 0.3 mole ergosterol per mole phospholipid phosphorus as analysed by gas chromatography, it is presumed that Amphotericin B is more active to ergosterol than to cholesterol. This would suggest that different degrees of membrane alteration induced by polyene antibiotics may reflect varying affinities of the antibiotics for different sterols. Therefore, it is of great purpose that Amphotericin B has been widely employed as an effective antifungal agent for treatment of various disease caused by fungi whose major sterol is ergosterol.

Furthermore, the remarked alterations by Amphotericin B in membranes were also confirmed by thin-sectioning electron microscopy (Fig. 3), demonstrating that in the Amphotericin B-treated *Epidermophyton* cell a particular phenomenon, "vesiculation" of the plasma membrane lying adjacent to cell wall was seen in many places, but no significant alteration was observed in other membrane structures. It can be strongly suggested that such severe damage caused by Amphotericin B would easily lead to leakage of cellular constituents followed by ultimate death of the cell. At present we think that the depressions observed on the fracture face may be related in some fashion with "vesiculation" of the plasma membrane, although more detailed investigations should be done to clarify the biological significance and formation mechanism of these depressions.

From the results presented above it may be suggested that ergosterol is primarily

localised in the plasma membrane of fungal cells and acts as the membrane stabilizer like cholesterol in mammalian membranes.

ADDENDUM (Received June 10th, 1974)

After this manuscript had been submitted, de Kruijff and Demel [30] proposed a model of Amphotericin B–cholesterol complex suggesting a circular arrangement of 8 Amphotericin B molecules interdigitated by 8 cholesterol molecules.

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