ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1985, p. 643–647
0066-4804/85/110643-05$02.00/0

Vol. 28, No. 5

Effect of the Imidazole Derivative Lombazole on the Ultrastructure of *Staphylococcus epidermidis* and *Candida albicans*

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Received 26 March 1985/Accepted 20 August 1985

Lombazole, an antimicrobial agent of the imidazole class, induced profound ultrastructural changes in *Staphylococcus epidermidis* and *Candida albicans*, as observed by freeze fracture electron microscopy. In *S. epidermidis* cells, the primary effect on ultrastructure was characterized by a distinct change in the morphology of the plasma membrane. Secondary effects of lombazole were cell wall thickening, accumulations of lipidlike material, abnormal cell division, severe change of shape, separation of the plasma membrane from the cell wall, and disruption of cells. The alterations in *C. albicans* were characterized by the deformation of and a decrease in the number of invaginations in the protoplasmic fracture face and corresponding ridges on the exoplasmic fracture face and by separation of the plasma membrane from the cell wall, leaving a gap which frequently contained small vesicles. Moreover, a considerable thickening of the cell wall occurred at localized regions. These structural alterations are discussed in relation to biochemical changes which may correlate with these phenomena.

Lombazole (Bay h 6020; 4-biphenylyl-(2-chlorophenyl)-1-imidazolyl methane) (Fig. 1) is an antimicrobial agent used in the treatment of acne (7). Like other members of the imidazole series, such as clotrimazole, miconazole, and bifonazole (28), the compound has a broad spectrum of activity. It is active against several budding and filamentous fungi as well as gram-positive bacteria.

In the same way as other imidazole antimycotics, lombazole most probably interferes with fungal lipid synthesis by inhibiting sterol C-14 demethylation (3). Since sterols are of limited occurrence in most bacteria (19), it is difficult to believe that the antibacterial activity of lombazole originates from interference with sterol synthesis. However, a preliminary study has indicated that lipid biosynthesis is the primary site of action of lombazole in the bacterium *Staphylococcus epidermidis* (D. Barug and H. B. Bastiaanse, Antonie Leeuwenhoek J. Microbiol., in press). Of the major metabolic processes investigated, the toxicant exerted its most pronounced effect on the incorporation of radiolabeled precursor into lipid fractions, of which phosphatidylglycerol was the main component. This occurred well in advance of an effect on growth.

Since the structural integrity of cell membranes is largely determined by lipids (6, 16), it is not surprising that profound alterations in membrane sturctures have been reported for various fungi after treatment with imidazole antimycotics (2, 5, 11, 21). As yet, morphological consequences of the action of imidazole derivatives on susceptible bacteria have not been studied. The present freeze fracture study was undertaken to determine time- and concentration-related changes in the ultrastructure of *S. epidermidis* after treatment with lombazole. For comparison, the effect of this toxicant on the ultrastructure of yeast-phase cells of *Candida albicans* was investigated. In addition to biochemical studies, these morphological investigations may be helpful for a better understanding of the mode of action of lombazole.

MATERIALS AND METHODS

Microorganisms and growth conditions. *S. epidermidis* ATCC 25167 was grown at 37°C in a synthetic amino acid medium (12). Cultures (200 ml in 500-ml conical flasks) were incubated in a gyratory water bath shaker. *C. albicans* (obtained from Bayer AG, Wuppertal, Federal Republic of Germany) was grown in shake cultures as described previously (1) with Kimmig broth nutrient medium.

Treatment with lombazole. Pure lombazole (generously supplied by Bayer AG) was added to exponentially growing cultures as a solution in acetone (final concentration of acetone, ≤0.05%). The same concentration of solvent was added to controls. Growth was monitored by measuring the optical density of the cultures at 535 nm. After various periods of incubation in the presence of several concentrations of lombazole (see Fig. 2 and 3), the effect of the toxicant on the ultrastructure of *S. epidermidis* and *C. albicans* was examined by freeze fracture electron microscopy.

Freeze fracture electron microscopy. Cells of untreated and lombazole-treated suspensions of *S. epidermidis* and *C. albicans* were harvested by centrifugation, washed twice, and fixed in 2% glutaraldehyde in Sörensen buffer (0.0666 M Na_2HPO_4-KH_2PO_4; pH 7.4) for 20 min at 0 to 4°C. Glutaraldehyde-fixed cells were suspended in 35% glycerol. Freeze fracture replication and electron microscopy were performed as described previously (2).

RESULTS

Effect on growth. The effect of several concentrations of lombazole on the growth of *S. epidermidis* and *C. albicans* in shake cultures is shown in Fig. 2 and 3.

Effects on the ultrastructure of *S. epidermidis*. The plasma membrane of untreated *S. epidermidis* cells incubated for 2,

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6, or 16 h was characterized by a taut appearance and the presence of intramembranous particles (Fig. 4A and B). The intramembranous particles on the protoplasmic fracture face (PF) greatly outnumbered those on the exoplasmic fracture face (EF). The cell wall appeared as a cross-fractured profile of uniform thickness and closely surrounded the fracture faces. In normal cell division, a cross-wall was formed in a centripetal manner, resulting in equal daughter cells.

The first morphological effects detectable in lombazole-treated *S. epidermidis* cells were found in the plasma membrane. Within 2 h, the taut appearance of the plasma membrane disappeared, revealing fracture faces with a bumpy appearance and vesicular invaginations (Fig. 4C). Even at the lowest concentration of lombazole (1 μg/ml) these changes were evident. The cell wall seemed to be morphologically unaltered. Virtually all cell divisions were occurring as in the untreated cells.

Further treatment (up to 6 h) resulted in additional effects of lombazole at 5 and 10 μg/ml. A considerable thickening of the cell wall was most conspicuous (Fig. 4D). The cross-walls did not show such an increase in thickness. Most of the treated cells showed a normal division process with centripetal cross-wall formation. Occasionally, cells with multiple cross-walls were observed; they may have resulted from cross-wall formation before the daughter cells from the previous division had separated.

After treatment for 16 h with 1 μg of lombazole per ml, the structural changes in *S. epidermidis* were still restricted to the plasma membrane. At increased concentrations of lombazole (5 and 10 μg/ml), alterations similar to those described above were noted. Furthermore, accumulations of material with the typical polymorphic appearance of freeze-fractured lipid (26) were noted in several of the treated cells (Fig. 4E). Abnormal cell divisions resulting in unequal daughter cells were also observed (Fig. 4E). Considerable changes of shape, separation of the plasma membrane from the cell wall, and even disruption of cells were frequently seen (Fig. 4E and F). The most severe structural changes could be observed after treatment with 10 μg of lombazole per ml.

Effects on the ultrastructure of *C. albicans*. The plasma membrane of untreated *C. albicans* cells appeared to be slightly undulating and was characterized by the presence of regularly sized, trough-shaped invaginations in the PF with corresponding ridges on the EF and an asymmetric distribution of intramembranous particles between the two fracture faces (Fig. 5A and B). The cell wall was very uniform in thickness, closely surrounding the plasma membrane (Fig. 5C).

After treatment of *C. albicans* cells with lombazole (4 μg/ml) for 24 h, profound alterations in the ultrastructure were observed (Fig. 5D and E). Invaginations and ridges strongly decreased in number and became deformed. The undulating appearance of the plasma membrane disappeared; the fracture faces showed a more taut appearance. In many cases, the plasma membrane and the cell wall did not fit closely together, leaving a gap which frequently contained small vesicles (Fig. 5F). A considerable local thickening of the cell wall was often observed (Fig. 5E).

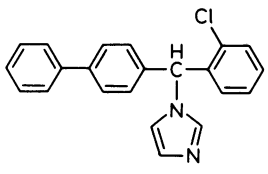

FIG. 1. Chemical structure of lombazole ($C_{22}H_{17}ClN_2$; molecular weight, 344.5).

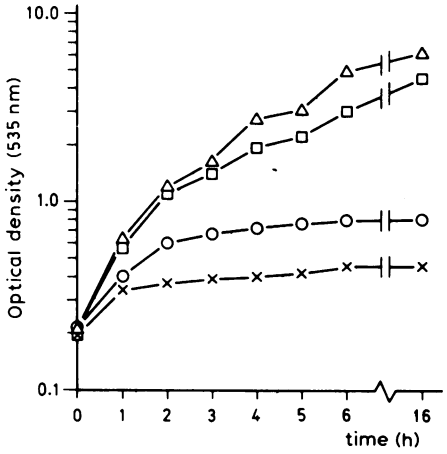

FIG. 2. Effect of lombazole on the growth of *S. epidermidis*. Solvent (control) or lombazole was added at zero time to exponentially growing cells, and growth was monitored by measuring the optical density. Symbols: △, control; □, lombazole at 1 μg/ml; ○, lombazole at 5 μg/ml; ×, lombazole at 10 μg/ml.

In experiments with lower concentrations of lombazole (1 and 2 μg/ml) or with a shorter incubation period (6 h), all the phenomena described above were recognized. However, they appeared to be less pronounced.

DISCUSSION

Electron-microscopic studies of ultrastructural alterations induced by imidazole derivatives have been described only for fungi (2, 5, 11, 21); they do not appear to have been reported for bacteria susceptible to these compounds. In lombazole-treated *S. epidermidis* cells, the primary morphological effect was seen in the plasma membrane, i.e., a bumpy appearance and the presence of vesicular invaginations. At present it is not possible to identify a cellular

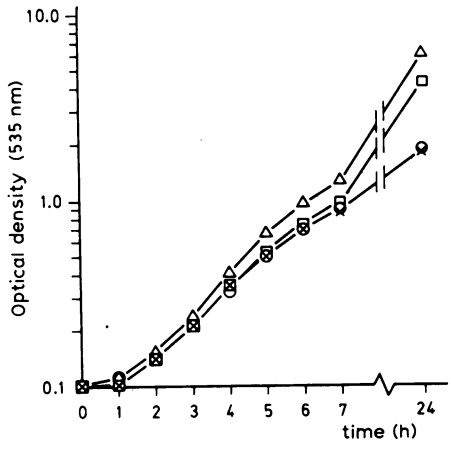

FIG. 3. Effect of lombazole on the growth of *C. albicans*. Solvent (control) or lombazole was added at zero time to exponentially growing cells, and growth was monitored by measuring the optical density. Symbols: △, control; □, lombazole at 1 μg/ml; ○, lombazole at 2 μg/ml; ×, lombazole at 4 μg/ml.

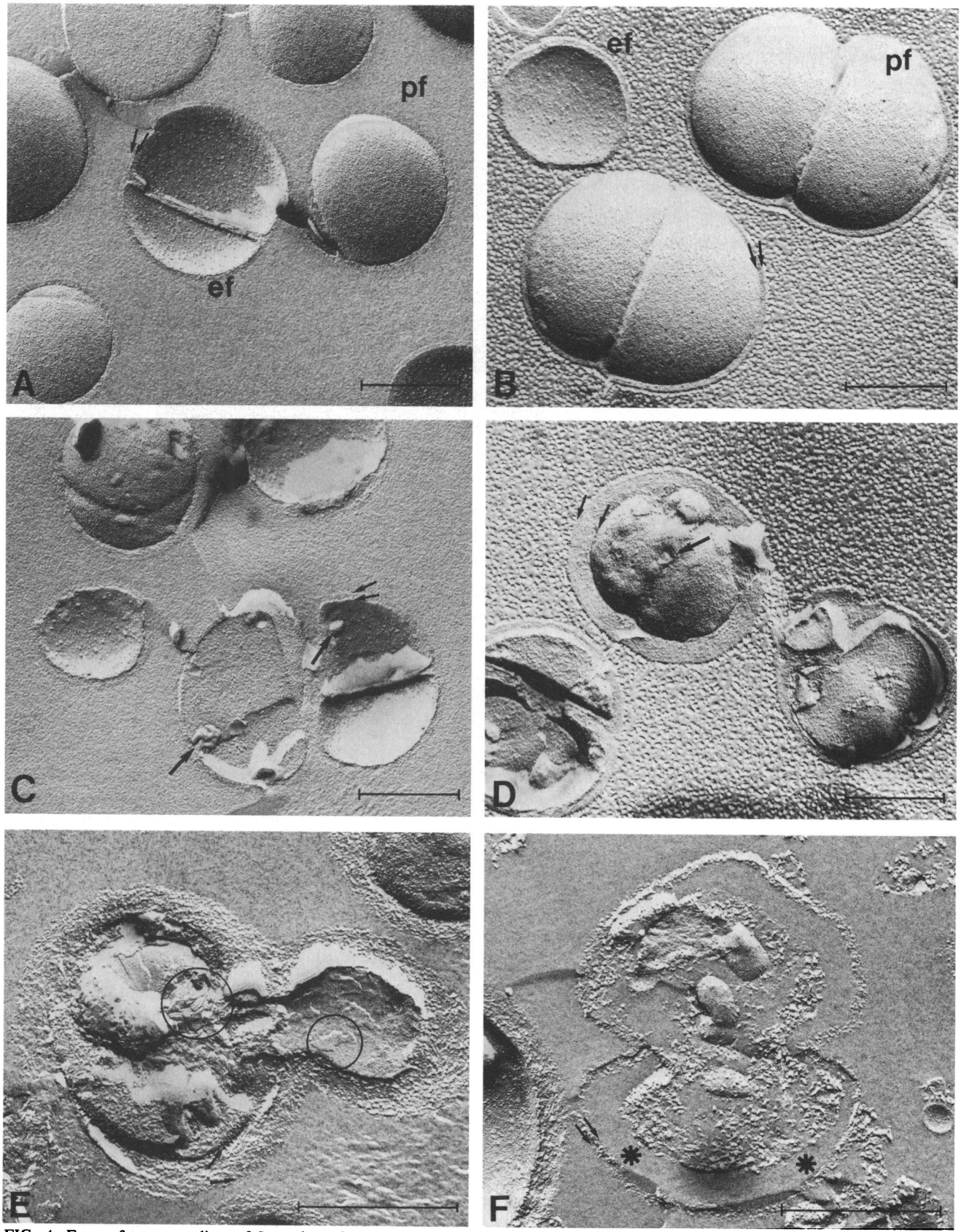

FIG. 4. Freeze fracture replicas of *S. epidermidis* cells grown for various periods in the absence or presence of lombazole. (A) Control, 2 h; (B) control, 6h; (C) lombazole at 5 μg/ml, 2 h; (D) lombazole at 5 μg/ml, 6 h; (E and F) lombazole at 10 μg/ml, 16 h. EF (ef) and PF (pf) can be recognized. Small arrows indicate cell wall thickness; larger arrows point to sites of bumpy appearance and vesicular invaginations. Lipidlike material (encircled) can be observed in panel E, and separation of the plasma membrane from the cell wall can be observed in panel F (asterisks). Photographs were mounted with Pt-C shadow casting from bottom to top. Bars, 0.5 μm.

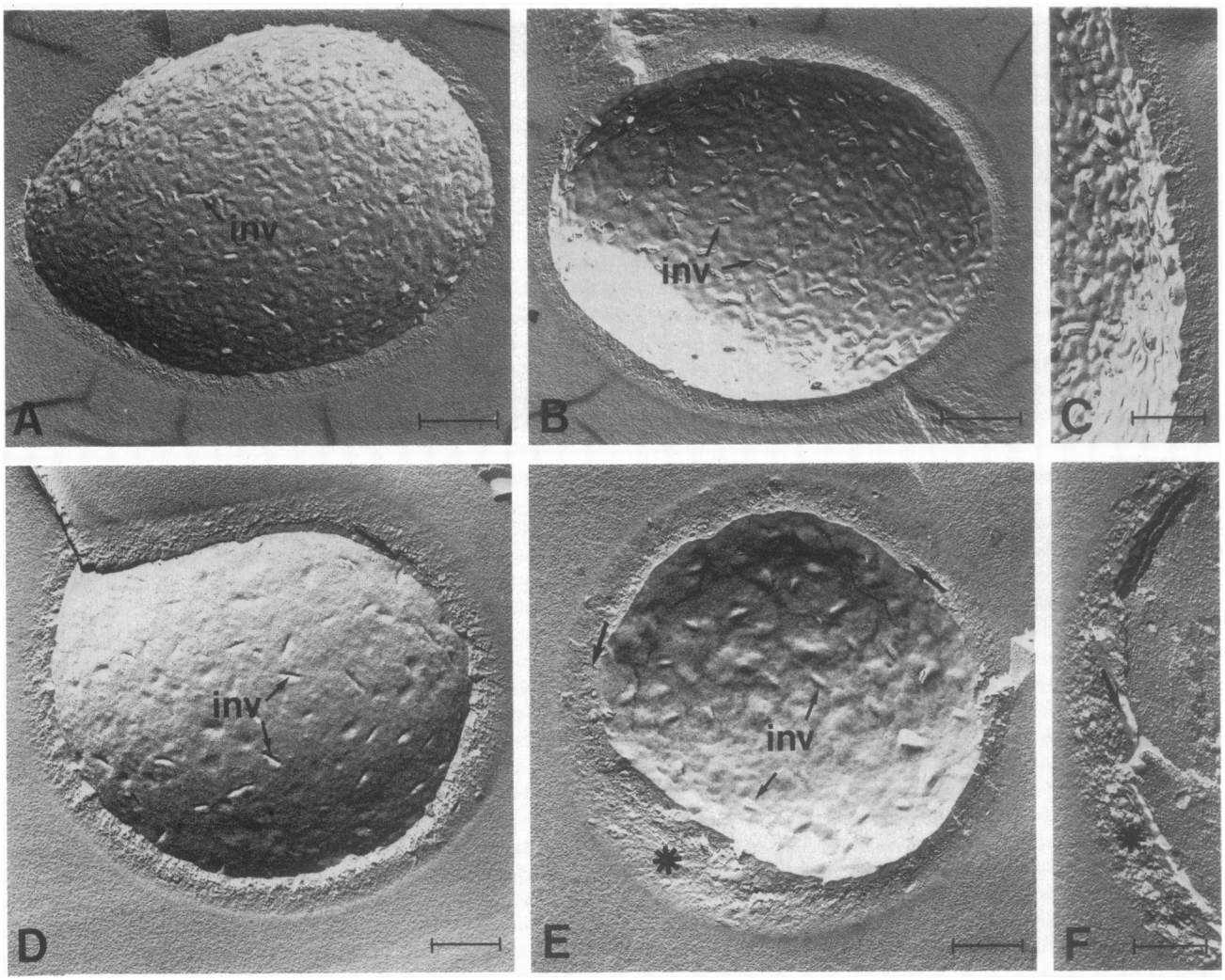

FIG. 5. Freeze fracture replicas of *C. albicans* cells grown for 24 h in the absence (A through C) or presence (D through F) of lombazole (4 μg/ml). PF (A and D), EF (B and E), and details of the cross-fractured cell wall (C and F) are shown. Note the decrease in the number of trough-shaped invaginations and ridges (inv), the local thickening of the cell wall (asterisks), and the separation of the plasma membrane from the cell wall, leaving a gap (large arrows) in lombazole-treated cells. Photographs were mounted with Pt-C shadow casting from bottom to top. Bars, 0.2 μm in panels C and F and 0.5 μm in the other panels.

biological basis for this phenomenon. It might be, at least partly, a reflection of the interference of lombazole with bacterial lipid biosynthesis.

Lombazole also induced additional morphological changes in *S. epidermidis*, i.e., cell wall thickening over the entire cell wall surface, accumulations of lipidlike material, abnormal cell divisions resulting in unequal daughter cells and, as final stages, separation of the plasma membrane from the cell wall and disruption of cells. These effects are considered to be secondary; they demonstrate the time- and concentration-dependent action of the toxicant.

Cell wall thickening has been reported to be a morphological marker of the disturbance of protein biosynthesis through nutritional deprivation or through the presence of inhibitors in members of various genera of bacteria (10, 17, 18, 22). In staphylococci, the cell wall contains numerous sites of peptidoglycan synthesis. Some sites are related to linear wall extension and are susceptible to the inhibition of protein synthesis; others are related to cell wall thickening and are independent of normal protein synthesis (23, 25). So, the increase in the cell wall thickness of *S. epidermidis* cells treated with lombazole might result from the inhibition of linear wall extension through a secondary effect of the toxicant on protein biosynthesis.

The abnormal cell divisions which were noted in *S. epidermidis* after treatment with increasing concentrations of lombazole for extended incubation periods indicate a disturbance of the regulation of cell division. The unequal daughter cells thus formed might result from interference with the centripetal growth of the cross-wall, as observed in staphylococci after treatment with chloramphenicol, actinomycin, or nitrofuran derivatives (10, 15), or from the presence of starting points of cross-wall formation not located at the coccal equator.

In *C. albicans* cells, the lombazole-induced alterations closely resembled those caused by its analog bifonazole (2). Particularly, the deformation of and a decrease in the number of invaginations in the PF and corresponding ridges on the EF and the separation of the plasma membrane from the cell wall, leaving a gap which frequently contained small vesicles, were noted. Comparable ultrastructural changes have also been shown by freeze fracture electron-micro-

scopic studies of fungi treated with the imidazole derivatives econazole and imazalil (11, 21). These alterations might be, at least partly, a reflection of the effects of this class of compounds on fungal sterol synthesis.

Apart from the above-mentioned ultrastructural changes, lombazole induced a considerable thickening of the cell wall of *C. albicans*. This might be caused by an abnormal deposition of the polymer chitin at localized areas. In budding yeasts, the formation of chitin, the major constituent of the primary septum, is initiated at a well-defined location in the cell and at a precise time in the cell cycle (8). Kerkenaar and Barug (14) have demonstrated that the inhibition of sterol C-14 demethylation in fungi is correlated with an abnormal deposition of chitin. Consequently, the regions of increased cell wall thickness in lombazole-treated *C. albicans* cells might indicate the locations at which new buds would have been formed had not the process been indirectly disturbed as a result of sterol biosynthesis inhibition by the toxicant.

Thickening of the cell wall of fungi treated with imidazole antimycotics has been described by other authors as well (9, 11, 20, 27). As mentioned above for lombazole, this phenomenon might be explained on the basis of an abnormal deposition of chitin. However, for some of these compounds an indirect effect on protein biosynthesis has been reported (13, 24). Although not so well documented as for bacteria, interference with protein biosynthesis in fungi also seems to result in a thickening of the cell wall (4). Therefore, the observed increase in the cell wall thickness might also be based on such an effect.

In conclusion, this freeze fracture electron-microscopic study has shown that the presence of lombazole primarily leads to a disturbance of the plasma membrane structure of both *S. epidermidis* and *C. albicans*. These findings correlate well with biochemical investigations which have indicated that interference with lipid biosynthesis is the primary effect of this toxicant (3; Barug and Bastiaanse, in press). A comprehensive paper on its antimicrobial mode of action is in preparation.

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