

## Protection by Unsaturated Lecithin Against the Imidazole Antimycotics, Clotrimazole and Miconazole

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The activity of egg lecithin in preventing the antifungal action of the two imidazole antimycotics, clotrimazole and miconazole, was confirmed. However, addition of this phospholipid could not relieve an existing imidazole inhibition. Compared with egg lecithin, reduced egg lecithin showed no such protective effect. The addition of egg lecithin to an aqueous suspension of the imidazole drugs changed the absorption profile of the imidazole, suggesting a low solubility and, consequently, a lower effective concentration; however, the addition of reduced egg lecithin did not produce any change in the adsorption. These results indicate that the preventive effect of egg lecithin on imidazole inhibition may be a consequence of preferential *in vitro* interaction of the drug with unsaturated phospholipid to form a hydrophobic complex.

Clotrimazole and miconazole are new imidazole derivatives that show a marked activity against yeasts, fungi, and some gram-positive bacteria (11, 13). Several papers dealing with the mechanism of imidazole action have demonstrated that both clotrimazole and miconazole apparently disturb the permeability characteristics of the cell membrane, which allows, on the one hand, leakage of essential precursors, metabolites, ions, and other intracellular components (7, 8, 12) and which produces, on the other hand, retardation of uptake of some amino acids from the medium (15). Our previous studies have shown that the anti-*Candida* activity of the two imidazole drugs is antagonized by several classes of lipids (e.g., phospholipids and acylglycerides), containing one or more acyl groups in its molecule, and by unsaturated fatty acids and that, unlike polyene antibiotics, none of the imidazoles can interact with cholesterol or ergosterol (14). These results have led us to postulate that the mechanism of antifungal action of imidazole involves an interaction with unsaturated phospholipids that are locating in cellular membranes, which causes these alterations in membrane permeability.

The present communication expands upon the observations made by measuring the antagonistic action of egg lecithin compared with that of reduced egg lecithin, which represented the unsaturated and saturated phospholipids, respectively, against both clotrimazole and miconazole on *C. albicans*.

### MATERIALS AND METHODS

**Organism.** *C. albicans* MTU 12021 was employed in this study. This strain was originally isolated from a patient with *Candida* vaginitis. Stock cultures were maintained by transfer at 2-month intervals on Sabouraud glucose agar.

**Chemicals.** Clotrimazole was furnished by Bayer Yakuhin Co., Ltd. (Osaka), and miconazole was obtained from Mochida Pharmaceutical Co., Ltd. (Tokyo). Stock solutions of each drug (8 mg/ml) were made with dimethyl sulfoxide and stored at  $-20^{\circ}\text{C}$ . All of the lecithins used were purchased from P-L Biochemicals Inc. A solution of lecithin was made with chloroform-methanol (2:1, vol/vol) or absolute ethanol as required. L-[ $U-^{14}\text{C}$ ]leucine (270 mCi/mmol) was obtained from Dai-ichi Pure Chemicals Co. (Tokyo).

**Cell viability.** Growth studies were made in a synthetic medium consisting of yeast nitrogen base broth (Difco) with 1% (wt/vol) glucose and 0.15% (wt/vol) L-asparagine, adjusted to pH 4.5. The yeast inoculum consisted of 5 ml of log-phase culture (6 to 9 h) grown at  $37^{\circ}\text{C}$  in shake flasks and diluted in the same medium to give a final optical density at 560 nm ( $\text{OD}_{560}$ ) of 0.40. Turbidity was measured by a Perkin-Elmer UV-VIS spectrophotometer equipped with a 10-mm light-path quartz cuvettes. An optical density of 0.10 was found to represent  $3 \times 10^8$  to  $4 \times 10^8$  viable cells per ml. A  $2\text{-}\mu\text{g/ml}$  portion of clotrimazole or miconazole prepared in a 1.0-ml volume of the synthetic medium was added to 1.0 ml of the same medium inoculated with a growing culture (1:1,000). Imidazole and lecithin were dissolved in dimethyl sulfoxide and chloroform-methanol, respectively. All the tubes containing imidazole and/or lecithin also contained the same concentration of the former solvent (1%, vol/vol) and the latter (2.5%, vol/vol) as did the

appropriate control tubes. In no case did the amounts of the two solvents inhibit the growth of *C. albicans* under study. Each tube was incubated statically at 37°C. Where indicated, 2 mg of either egg lecithin or reduced egg lecithin per ml (in a volume of 0.05 ml) was supplemented at zero time or at 24 h, and incubation was further continued. After the onset of incubation, at 24 and 48 h, samples were withdrawn and viability was determined by plating dilutions (in Sabouraud glucose broth) of yeast cultures on Sabouraud glucose agar. The plates were incubated at 37°C for 48 h before the colonies were counted.

**Uptake of radioactively labeled leucine.** Tests for growth and starvation of *C. albicans* cells were performed according to the same procedures as described previously (15). The indicated amounts of imidazole (30 µg/ml) and/or lecithin (50 µg/ml) were added to starved cells suspended in a 0.4-mM KH<sub>2</sub>PO<sub>4</sub> solution. With or without subsequent incubation at 37°C for 60 min, [<sup>14</sup>C]leucine, to a concentration of 1 mM, was added to each reaction mixture, and further incubation was made. After 2 h at 37°C, a 2-ml volume of samples was taken, and the reaction was immediately stopped with 1 ml of 3 × 10<sup>-3</sup> M uranyl nitrate (pH 4.0). The cells were harvested by centrifugation, washed three times in 10<sup>-3</sup> M uranyl nitrate in a total volume of 15 ml, and then extracted with 2 ml of 95% (vol/vol) ethanol at 25°C for 30 min. A portion of the extract removed by centrifugation was placed in a counting vial, and the radioactivity was measured on a Packard Tricarb liquid scintillation spectrophotometer 2425 series.

**Spectral studies.** Clotrimazole and miconazole were dissolved in absolute ethanol, diluted 50-fold with 0.1 M succinate-phosphate buffer (pH 4.0), and employed at a final drug concentration of 25 µg/ml. Lecithins were also dissolved in ethanol and dispersed in the same buffer by vigorous vibration in a Vortex mixer. Subsequently, reactions between imidazole and lecithin were carried out in a 4-ml volume of 2% (vol/vol) ethanol. After incubation at 25°C for 2 h, imidazole samples with or without lecithin were taken to measure the absorption spectrum from 240 to 290 nm in a Shimadzu multipurpose spectrophotometer model MPS-5000. Spectral analysis of the reaction combinations of imidazole and lecithin was compared with that of lecithin alone.

## RESULTS

**Growth-inhibitory effect.** Under the present experimental conditions where the viable count for untreated control cultures increased 300- and 900-fold during the incubation period of 24 and 48 h, respectively, addition of 1 µg of clotrimazole or miconazole per ml to these cultures at zero time led to a gradual decrease in the viable count, reaching a value 10 times as low as the initial one after 48 h of incubation (Fig. 1). On the other hand, lecithin was added at too low a concentration (50 µg/ml) to affect the yeast growth when used alone. When egg lecithin was added at zero time along with clotrimazole or miconazole, there was 10- to 50-fold

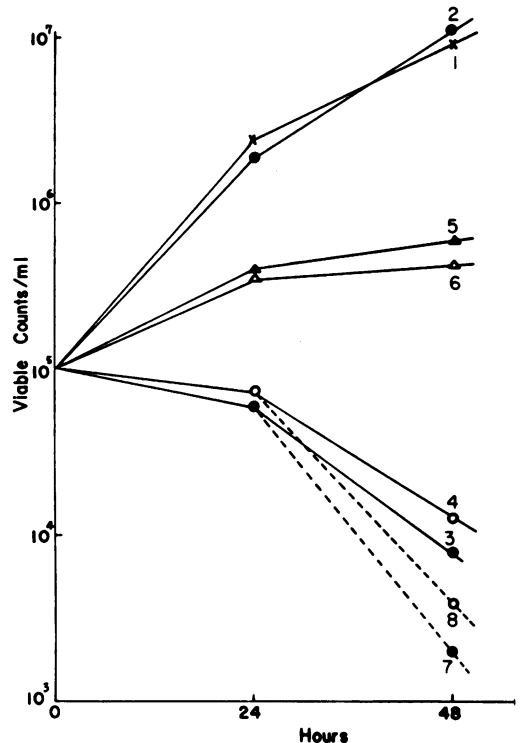


FIG. 1. Effect of egg lecithin on anti-*Candida* activity of imidazole antimycotics. To *C. albicans* cultures were added egg lecithin (50 µg/ml) and imidazole (1 µg/ml), alone and in combination, at zero time. (1) No addition; (2) lecithin; (3) clotrimazole; (4) miconazole; (5) clotrimazole plus lecithin; and (6) miconazole plus lecithin. Otherwise, egg lecithin was added to yeast cultures 24 h after they had been preincubated with (7) clotrimazole or (8) miconazole.

restoration in the viable count as compared with the value for the comparable cultures, which had been exposed to imidazole alone. In contrast, no such protective effect was noted with reduced egg lecithin (data not shown).

Experiments were also performed to learn whether egg lecithin was effective in reversing inhibition by the imidazole drugs even when the cells were treated with either clotrimazole or miconazole for 24 h before the addition of the phospholipid. Egg lecithin produced no reversion of the antifungal action of the two imidazole drugs (Fig. 1).

**Effect of leucine uptake.** Both clotrimazole and miconazole at a concentration of 30 µg/ml almost completely inhibited the entry and accumulation of leucine in the amino acid pool in starved *C. albicans* cells (Table 1). When egg lecithin (50 µg/ml) was added to the test mixture either before or with addition of clotrimazole (30 µg/ml) at zero time, the inhibition of leucine

uptake was partially prevented. However, the addition of egg lecithin to cells already inhibited by clotrimazole (at 60 min) did not alter the effect of the imidazole drug. Comparable results were obtained with 30  $\mu\text{g}$  of miconazole per ml. Compared with egg lecithin, reduced egg lecithin

TABLE 1. Uptake of leucine by *C. albicans* cells preincubated for 60 min with imidazole and lecithin, alone and in combination<sup>a</sup>

Treatment	Leucine taken up (nmol/mg [dry wt] of cells)
None	27.8
Egg lecithin	26.1
Reduced egg lecithin	27.5
Clotrimazole	0.4
Clotrimazole + egg lecithin	9.4
Clotrimazole + egg lecithin <sup>b</sup>	0.6
Clotrimazole + reduced egg lecithin	0.1
Clotrimazole + reduced egg lecithin <sup>b</sup>	0.1
Miconazole	0.2
Miconazole + egg lecithin	6.8
Miconazole + egg lecithin <sup>b</sup>	0.2
Miconazole + reduced egg lecithin	0.1
Miconazole + reduced egg lecithin <sup>b</sup>	0.2

<sup>a</sup> Final concentrations of imidazole and lecithin were 30 and 50  $\mu\text{g}/\text{ml}$ , respectively.

<sup>b</sup> Lecithin was added at zero time to cells that had been preincubated for 60 min with the indicated imidazole.

did not show any protection against the action of the two imidazole drugs.

**Influence on the absorption spectrum of imidazoles.** When the two imidazole drugs were incubated in citrate-phosphate buffer containing egg lecithin, their absorbance values increased significantly at wavelengths ranging from 240 to 290 nm, and, concomitantly, the absorption peak at 260 nm for clotrimazole and at 265 nm for miconazole became less prominent (Fig. 2). The indicated changes were intensified during longer incubation periods. Such a general increase in the absorbance value for the two imidazole drugs without a major shift in the character of the spectrum may be due to a fine amorphous precipitate; this increase most likely represents aggregation of colloidal imidazole by egg lecithin. In contrast, reduced egg lecithin had no effect on the absorbance value of the two imidazole drugs.

## DISCUSSION

The present study expands upon our previous microbiological experiments, in which the apparent antagonistic effect on the inhibitory action of the imidazole antimycotics was demonstrated for naturally occurring phospholipids (e.g., egg lecithin) and synthetic unsaturated

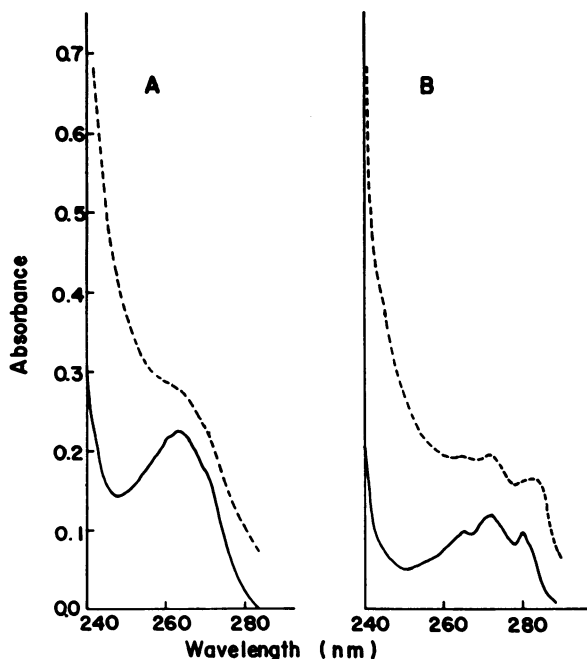


FIG. 2. Absorption spectra of clotrimazole (A) and miconazole (B) in the presence (---) and absence (—) of egg lecithin. Final concentrations of imidazole and lecithin in the reaction mixture were 50 and 25  $\mu\text{g}/\text{ml}$ , respectively.

lecithins, but not for reduced egg lecithin or any of the saturated phospholipids tested (14). These data led us to propose that the unsaturated acyl group in the molecule of lecithin (and other types of phospholipids) along with the imidazole drugs, may play a principal role in exerting an antagonistic effect. Moreover, from the results presented here based on growth, amino acid uptake, and spectral measurements, we suggest that although egg lecithin is effective in preventing imidazole inhibition, it is incapable of reversing an existing drug effect and that purely physicochemical factors are involved in this phospholipid action. Thus, the antagonistic effect of egg lecithin may have resulted from the occurrence of a physicochemical interaction between the phospholipid and the imidazole drug so that access of the latter to the target site on the cell membrane can be affected. On the other hand, it is less likely that imidazole can inhibit synthesis of some unsaturated lecithins or fatty acids by *C. albicans* cells. The spectral analyses demonstrate a physicochemical interaction between the imidazole drugs and egg lecithin (but not reduced egg lecithin) to form hydrophobic complexes. This action may result in a lowering of the effective drug concentration in the medium.

Several other membrane-active antifungal agents have also been shown to be counteracted by certain classes of lipid compounds. Other studies have demonstrated that the inhibition by polyene antibiotics (e.g., nystatin and filipin) is protected by the addition of sterols to the test medium (2, 3, 9, 16). It has also been found that several different biological actions of the polypeptide antifungal antibiotic, mycobacillin, toward sensitive fungi are antagonized by both sterols and egg lecithin (4-6). It appears from these results that the mechanism of membrane action of the imidazole drugs is different from that of polyene antibiotics or mycobacillin in that the former drugs have a preferential affinity for unsaturated lecithins.

It is generally accepted that phospholipids constituting the cell membrane play an important role in membrane physiology. Combs et al. (1) and Nishi et al. (10) found that the phospholipid composition of *C. albicans* cells is characterized by a relatively high proportion of unsaturated members including lecithins. Therefore, the potent anti-*Candida* action of the imidazole antimycotics might be accounted for by the plentiful existence of unsaturated phospholipids in cellular membranes of this susceptible organism,

which provide the preferential target molecule for the drug action.

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