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Effect of Ketoconazole on the Fungicidal Action of Amphotericin B in *Candida albicans*

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Amphotericin B-susceptible *Candida albicans* became resistant to the drug after growth in the presence of ketoconazole. Chromatographic analysis of cellular sterols showed that the organisms became depleted of ergosterol in parallel with the development of amphotericin B resistance. The implications of these findings are discussed in relation to combination chemotherapy with these two important antifungal agents.

To improve the suboptimal therapy for many fungal infections, the efficacy of some drug combinations has been examined. Several studies involving combinations of amphotericin B with other antimicrobial agents have been reported. Such combinations were expected to be synergistic because amphotericin B facilitated the entry of the second agent into the fungal cell; synergism of amphotericin B with 5-fluorocytosine (3, 10, 12, 16), rifampin (1, 10, 14), and tetracycline (8, 11) against various fungi has been observed. Combinations of amphotericin B with the antifungal imidazoles have given conflicting results; antagonism was reported with miconazole (2, 15), clotrimazole (2), and econazole (3), but the amphotericin B-ketoconazole combination was found to be somewhat superior to either of the drugs alone (4).

From what is known about the mechanisms of antifungal action of the polyenes and the imidazoles, the observed antagonism is not surprising. The polyene antifungals are fungicidal. They damage the cytoplasmic membrane of susceptible organisms. Under most conditions, membrane sterols are required for membrane susceptibility; $\Delta^{5,7}$-sterols such as ergosterol are more efficient in conferring sensitivity to the polyenes (5, 6) than is cholesterol, which has only one double bond in the B ring (Δ^5). Polyene-resistant mutants in fungi have been isolated and found to be of two types: those with normal and those with altered sterol composition (7, 9, 13). The mechanism of resistance in the former group is unknown. Fungi in the latter group are blocked at one of several steps in the synthesis of ergosterol from lanosterol. The antifungal imidazoles also inhibit ergosterol biosynthesis by blocking the C-14 demethylation of lanosterol, resulting in accumulation of methylated ergosterol precursors (17, 19). It is clear that these agents may alter the susceptibility of organisms to polyenes. The following experiments show that *Candida albicans* developed resistance to amphotericin B after growth in the presence of the imidazole ketoconazole; cellular ergosterol decreased in parallel.

C. albicans, a clinical isolate, was grown in broth (1% tryptone–0.5% yeast extract–2% glucose) with aeration at 35°C. The medium used for growing the overnight inocula and for the drug interaction experiments contained sodium [2-^{14}C]acetate (0.5 μCi/ml of medium supplemented with cold sodium acetate; final concentration, 10 μg ml^{-1}). An overnight culture was diluted 10-fold into fresh medium, and the incubation was continued. At time zero, the culture was again diluted into fresh, prewarmed medium to give a cell density of approximately 10^6 cells ml^{-1}. One-half of the culture was transferred to a flask containing ketoconazole (final concentration, 3.3×10^{-7} M) in dimethyl sulfoxide. To the other half, an equivalent amount of dimethyl sulfoxide was added; this culture served as the control. The incubation was continued for 5 h, and hourly samples were withdrawn for (i) isolation of sterols as previously described (17), (ii) enumeration of cells in a Petroff-Hausser chamber, (iii) viable counts by plating of appropriate dilutions, and (iv) determination of amphotericin B susceptibility. The latter was done by exposing the cells to amphotericin B (5 μg ml^{-1}) for 15 min, followed by viable count determination, using the standard plating technique. Before exposure to amphotericin B, all samples were adjusted to a cell density of 10^6 cells ml^{-1}.

The non-saponifiable fractions containing the sterol were fractionated by high-pressure liquid chromatography, using a Waters Associates (Milford, Mass.) model 6000A instrument equipped with a solvent delivery system (model

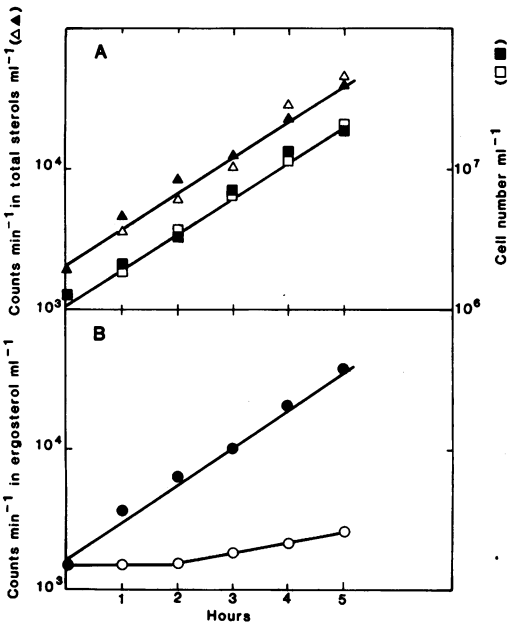

FIG. 1. Effect of ketoconazole on growth and sterol synthesis in *C. albicans*. Cells growing in the presence of [2-^{14}C]acetate were exposed to ketoconazole. At intervals, samples were taken for cell counts (Petroff-Hausser chamber) and for isolation of total sterols. Ergosterol was determined after fractionation of total sterols by high-pressure liquid chromatography. (A) Cell numbers (squares) and total sterol synthesis (triangles) in untreated (closed symbols) and ketoconazole-treated (open symbols) cultures. (B) Ergosterol synthesis in untreated culture (●) and ketoconazole-treated culture (○).

M-45), an injector (model U6K), and a detector (model 450) operated at 205 nm. The column was an ODS column (Altex Scientific, Inc.) (0.46 by 250 mm), and the eluting solvent was methanol at a flow rate of 0.6 ml min^{-1}. Authentic sterols were used for establishing retention times and elution profiles. Under the conditions employed, ergosterol eluted 15 to 17 min after injection. The fractions were collected directly in scintillation vials, and after the addition of 10 ml of Aquasol (New England Nuclear Corp., Boston, Mass.), radioactivity was determined in a Beckman liquid scintillation counter.

Ketoconazole, even at high concentrations (10^{-3} M), has been shown to be fungistatic and not fungicidal for *Saccharomyces cerevisiae* (18) and for most other fungi (unpublished data). At the concentration used in these experiments (3.3×10^{-7} M), ketoconazole had no effect on the growth of *C. albicans* over several hours, as judged by the rate of increase in the number of cells (Fig. 1A) and by the incorporation of ^{14}C into total sterol fraction (Fig. 1B). However, biosynthesis of ergosterol was completely inhibited within minutes (Fig. 1B). There was a concomitant increase in the content of lanosterol and other methylated sterols (data not shown).

The *C. albicans* isolate used in this study was highly susceptible to the fungicidal action of amphotericin B, showing a 4-log decrease in viability after treatment with 5 μg ml^{-1} for 15 min. In the ketoconazole-treated culture, however, amphotericin B-resistant cells appeared after 1 h and increased steadily, so that after 4 h more than 90% of the cells were refractory to amphotericin B (Fig. 2). Figure 2 also shows the ergosterol content of ketoconazole-treated cells relative to the control cells during the experiment. The rate of depletion of ergosterol content per cell with time roughly paralleled the loss of amphotericin B susceptibility after ketoconazole treatment. The experimental data support the hypothesis that the amphotericin B resistance in *C. albicans* which develops after exposure to ketoconazole is a direct consequence of ergosterol depletion.

Under the conditions of these experiments, growth in ketoconazole blocks the lethal action of amphotericin B. However, there is no reason to expect the converse, that amphotericin B interferes with the action of imidazoles. Hence, one would not anticipate that the combination of

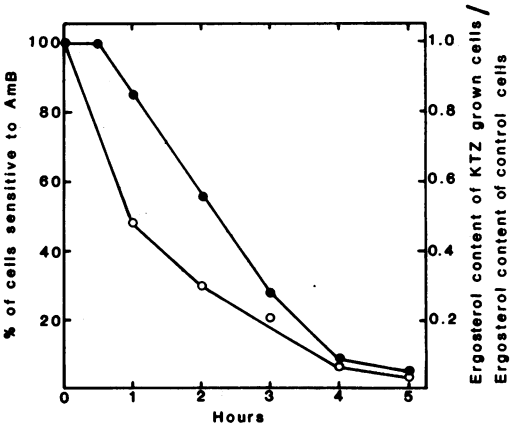

FIG. 2. Ergosterol content and amphotericin B susceptibility of *C. albicans* growing in the presence of ketoconazole. The plotted data on ergosterol content per cell were calculated from the results of the experiment shown in Fig. 1. Ergosterol content of ketoconazole-exposed culture is expressed as the ratio of ^{14}C counts in ergosterol fractions of ketoconazole-treated culture to similar fractions from the control culture corrected for changes in cell number. Amphotericin B susceptibility was determined by colony counts before and after exposure of cells to amphotericin B (5 μg ml^{-1}). Symbols: ●, amphotericin B; ○, ergosterol content.

amphotericin B and ketoconazole would be less effective than ketoconazole alone. However, the combination may have less fungicidal activity than amphotericin B alone. If one is interested in achieving a fungicidal therapeutic regimen, it would appear unwise to use this combination.

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