

SHORT COMMUNICATION

Disappearance of Nystatin Resistance in *Candida* Mediated by Ergosterol

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Two nystatin-resistant mutants of *Candida* were isolated *in vivo* and identified as *C. albicans* and *C. krusei*. Analysis of the sterol composition of these cells indicated a total absence of ergosterol and an increased level of a possible precursor. Successive cultures of the resistant strains in a medium supplemented with 10 µg ergosterol ml⁻¹ induced in 5 d a sensitivity to nystatin identical to that of the wild-type strain. These ergosterol-supplemented strains had an ergosterol content similar to that of the wild-type strain cultured in the absence of ergosterol. Resistance to nystatin was recovered after 5 d subculture in medium without ergosterol.

INTRODUCTION

Nystatin selectively attacks fungi by interacting with their membranes (Kruffy & Demel, 1974). Ergosterol, the major membrane sterol in yeast, has been identified as the binding site for nystatin (Lampen *et al.*, 1962; Norman *et al.*, 1972), and many studies have shown the formation of complexes between sterols and nystatin in biological membranes (Verkleij *et al.*, 1973; Kruffy & Demel, 1974). In the latter cases, nystatin leads to irreversible changes in membrane permeability and the loss of cell viability. In fungi, the development of resistance to nystatin in the laboratory is frequently associated with a decrease in the ergosterol content of the resistant cells (Thompson *et al.*, 1971; Woods, 1971; Fryberg *et al.*, 1975).

There have been several reports of an increase in the sensitivity of micro-organisms to polyene antifungals on sterol-containing media (Razin, 1963; Weber & Kinsky, 1965; Feingold, 1965; Karst & Lacroute, 1973; Razin, 1975). In some of these studies, the micro-organisms examined – *Mycoplasma laidlawii* (Razin, 1963; Weber & Kinsky, 1965; Feingold, 1965) and *Pythium* spp. (Schlosser & Gottlieb, 1966) – do not naturally contain, require or synthesize sterols. Thus, the *M. laidlawii* and *Pythium* cells are normally insensitive to polyene antibiotics, but when incubated in a cholesterol-supplemented medium acquire a 'transient' sensitivity to filipin (Lampen *et al.*, 1962; Weber & Kinsky, 1965) or amphotericin B (Feingold, 1965), the most aggressive of the antifungals, but not to nystatin.

In this paper, we report studies on two nystatin-resistant strains of *Candida* isolated *in vivo* which became sensitive to nystatin after incubation with ergosterol.

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METHODS

Organisms. The *Candida albicans* sensitive, *C. albicans* resistant and *C. krusei* resistant strains were isolated from three patients with candidiasis. Once isolated, each strain was purified, subcloned three times, identified by standard techniques and tested *in vitro* for its sensitivity to nystatin.

In our experiments, we compared the response to nystatin of the *C. albicans* sensitive and resistant strains. Some results are also included for the *C. krusei* resistant strain since it behaved in the same way as the *C. albicans* resistant strain.

Growth conditions. Stock cultures were maintained on Difco Sabouraud dextrose agar (SDA) at 5 °C. Where indicated, this medium was supplemented with either ergosterol (10 µg ml⁻¹) before autoclaving, or nystatin (2.2 to 143.4 µg ml⁻¹) after autoclaving, or both. The nystatin was dissolved in a 1 % (v/v) Tween 80 solution in absolute ethanol, and the ergosterol was kept as an ethanolic stock solution containing 40 mg ml⁻¹.

The inoculum was taken from a fresh overnight plate culture on SDA and incubated at 30 °C before each resistance analysis. Cultures for resistance analysis were grown on solid media for 40 to 70 h, subculturing each day on SDA or on SDA supplemented with ergosterol, before analysis. Minimum inhibitory concentrations (m.i.c.) were determined by the method of Hamilton-Miller (1972).

For sterol analysis and ergosterol determinations, the cells were grown on liquid Sabouraud dextrose medium for 24 h at 30 °C on a reciprocal shaker.

Sterol analysis. Non-saponifiable sterols were extracted by the method of Breivik & Owades (1957). Their absorption spectra between 190 and 310 nm were recorded with a Unicam recording spectrophotometer. For dry weight determinations, 200 mg (wet wt) cells were diluted in 10 ml 50 mM-phosphate buffer pH 7.0, filtered through pre-weighed Millipore filters (type HAWG 047SO), washed once with 3 ml water and reweighed after drying for 24 h at 85 °C. The relative percentages of sterol were calculated by the method of Breivik & Owades (1957).

RESULTS AND DISCUSSION

The original degree of resistance to nystatin of the *C. albicans* and *C. krusei* resistant strains was 16 and 8 times higher, respectively, than that of the *C. albicans* sensitive strain. After subculturing *in vitro*, a progressive loss of resistance to nystatin was developed but stable m.i.c. values were reached after subculturing for 10 months (Fig. 1*a*). The following experiments were performed once stable m.i.c. values were observed. When the *C. albicans* and *C. krusei* resistant strains were subcultured on ergosterol-supplemented medium, they showed a considerable decrease in their resistance to the antibiotic (Fig. 1*b, c*). This decrease in resistance was reversed and the original m.i.c. values were obtained after subculturing for 5 d on medium without ergosterol (Fig. 1*d*). The sensitive strain of *C. albicans* showed the same sensitivity to nystatin regardless of the presence of ergosterol in the subculture medium (Fig. 1*a, b, c*).

The absorption spectra of the sterol extracts from the resistant strains did not show the ergosterol absorption band, but rather an increase was detected in the squalene peak at 205 nm; thus they contained no ergosterol. In contrast, the extract from the *C. albicans* sensitive strain showed the absorption peaks for ergosterol at 271, 281 and 293 nm which are characteristic of the 5,7-diene system; the calculated ergosterol content was 1.7 % of dry weight. The absorption spectra of sterol extracts from the resistant strains after preculture for 72 h on ergosterol-supplemented medium showed the ergosterol band, a decreased squalene peak and a new peak at 220 nm which was not observed in the sensitive strain. The calculated ergosterol contents under these conditions were 1.8, 1.6 and 1.6 % of dry weight for the *C. albicans* sensitive, *C. albicans* resistant and *C. krusei* resistant strains, respectively.

Whereas subculture for 5 d in an ergosterol medium, each day starting with a small inoculum, was required to convert the *Candida* resistant strains to the nystatin-sensitive form (Fig. 1*c*), only 10 min was required for a similar conversion of *Mycoplasma laidlawii* to amphotericin B sensitivity (Feingold, 1965). Once *Candida* cells became nystatin-sensitive, they remained so for at least 3 d. In contrast, the reversal of growth inhibition occurred in 2 to 4 h for other macrolide antibiotics in *M. laidlawii* (Feingold, 1965) or in *Pythium* spp. (Schlosser & Gottlieb, 1966).

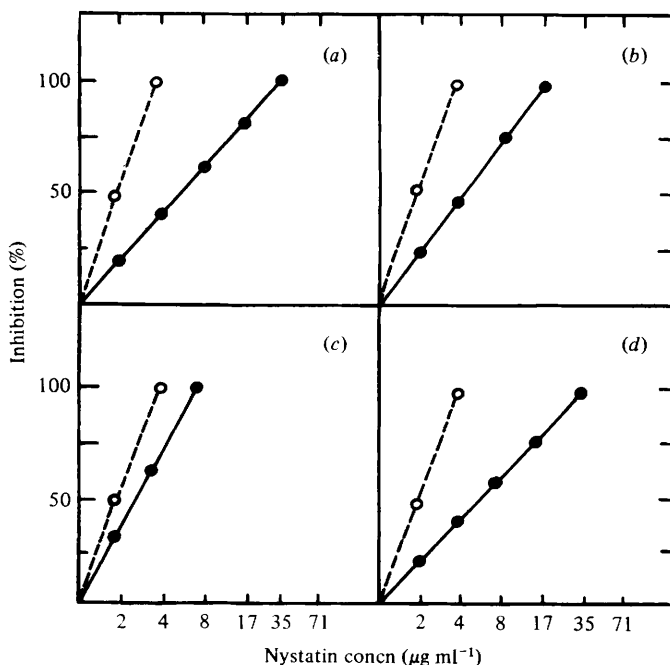


Fig. 1. Minimum inhibitory concentrations of nystatin for the *Candida albicans* sensitive (○) and resistant (●) strains. (a) Stabilization of the resistance to nystatin after subculturing *in vitro* for 10 months; (b) after subculturing for 48 h on medium supplemented with ergosterol; (c) after subculturing for 5 d on medium supplemented with ergosterol; (d) after subculturing for 5 d on unsupplemented medium. The response to nystatin of the *C. krusei* resistant strain was similar to that of the *C. albicans* resistant strain.

These differences are compatible with the idea that the incorporation of sterols into cell membranes can take place in at least two different functional sites. One of these binding sites, present in cell membranes which naturally lack sterol, accepts and loses the sterol rapidly and easily. The other binding site, present in cell membranes that naturally contain sterols, needs an active growth phase during which ergosterol is gradually incorporated and during which the disappearance of ergosterol is also progressive. In the first case, the effect of antibiotics is transient, while in the second, the effect is more prolonged. A particular phase of sterol assembly may be of importance in determining the role of sterols in cell membranes. Some reports supporting this idea show that the amount of sterol incorporated by cell membranes is determined by their phospholipid and total lipid content (Razin, 1974). Furthermore, due to the heterogeneous lipid composition of the membrane, cholesterol might not be homogeneously distributed in the membrane (Kruyff *et al.*, 1974). Indeed, it is known that in artificial bilayers the transfer of sterol from one side to the other side of the bilayer (flip-flop) is determined by the ratio of the different components that form the membrane (Smith & Green, 1974). The interactions between sterols and polyene antibiotics could therefore alter (Kruyff & Demel, 1974).

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