SHORT COMMUNICATION

Phenotypic Resistance to Miconazole and Amphotericin B in *Candida albicans*

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Phenotypic resistance to both amphotericin B and miconazole develops in stationary phase cultures of *Candida albicans* and this resistance lies in changes in the cell wall. Study of the effects of growth conditions, treatment with SH-reactive agents and treatment with enzymes indicates that the nature of the changes leading to resistance must be different for the two drugs.

INTRODUCTION

The effect of polyene antibiotics on *Candida albicans* is to induce a change in the permeability of the cytoplasmic membrane such that small molecular weight substances retained in the cytoplasm against a concentration gradient leak out of the organisms (Lampen, 1966). The leakage of potassium ions can be used to monitor the change in membrane permeability, and the rate of K⁺ leakage used as a measure of polyene sensitivity (Gale, 1974). When estimated in this way, the amphotericin sensitivity of *C. albicans* is found to vary with the phase of growth; exponentially growing organisms are highly sensitive but the sensitivity decreases as the culture enters the stationary phase until organisms that have been in the stationary phase for several days may have a resistance two or three orders of magnitude greater than that of exponentially growing organisms. Spheroplasts of resistant organisms have the same sensitivity as those from exponentially growing cells: the resistance of stationary phase cells must therefore lie in changes in the structure or organization of the cell wall (Gale *et al.*, 1975).

Another group of antifungal drugs which, amongst other actions, induce a change in membrane permeability is the imidazole series, of which clotrimazole (Plempel *et al.*, 1969) and miconazole (Van Cutsem & Thienpont, 1972) have been shown to produce leakage of cations and other small molecular weight substances from *C. albicans* (Iwata *et al.*, 1973; Swamy *et al.*, 1974). The leakage of K⁺ can be monitored in the same way as for polyene antibiotics (Gale, 1974) and the present paper shows that increases in resistance, similar to those that have been described for amphotericin, occur as cultures of *C. albicans* grow into stationary phase.

Stationary phase cultures of *C. albicans* thus have high resistance to both amphotericin and miconazole as measured by the release of K⁺. This paper is not a study of the mode of action of miconazole but is directed towards finding out whether resistance towards these two drugs, of dissimilar chemical structure, arises from the same cause in both cases. It is shown that, although resistance in both cases appears to lie in changes in the cell wall, variations in that resistance produced by alterations in reducing conditions, treatment with SH-reactive agents or with enzymes are sufficiently different to indicate that a common change is not involved.

METHODS

Organism and growth conditions. *Candida albicans* strain 6406, from the Mycological Reference Laboratory of the London School of Hygiene and Tropical Medicine, was maintained, cultured and prepared in washed suspension as previously described (Gale, 1974). For most of the experiments, cultures were grown at 37 °C in batches on a rotary incubator. For the experiments involving alterations in O₂ saturation during growth, the organism was grown in a fermenter under controlled conditions of temperature, pH, aeration and O₂ saturation as described by Gale *et al.* (1977).

Estimation of sensitivity by the K⁺ release method. In this paper the terms 'sensitivity' and 'resistance' refer to the action of either amphotericin B methyl ester (AME) or miconazole (MN) in inducing the release of K⁺ from suspensions of organisms, monitored by a K⁺-sensitive electrode (Gale, 1974). As previously described, the release of K⁺ after the addition of AME is both time- and concentration-dependent and sensitivity has been determined in terms of the standard release concentration (s.r.c.) defined as the concentration (in µg ml⁻¹) of AME required to induce a release (above that in the control without drug) equal to 1 nmol K⁺ min⁻¹ (mg dry wt organisms)⁻¹ in 8 min at 20 °C. With MN it was found that the rate of K⁺ release rapidly increased during the first 2 min after addition of the drug but then attained a steady rate varying with the MN concentration. For the experiments with MN, therefore, the sensitivity is defined as the concentration of MN (in µg ml⁻¹) required to induce a steady release of 1 nmol K⁺ min⁻¹ (mg dry wt organisms)⁻¹ above that in the control without drug.

Treatment with SH-reactive agents. This was carried out as described by Gale *et al.* (1975).

Digestion with enzymes; formation of spheroplasts. Treatment with enzymes was carried out as described by Gale *et al.* (1980) and the production of spheroplasts as described by Gale *et al.* (1975).

Chemicals. The enzyme preparations were the same as those described by Gale *et al.* (1980). Amphotericin B methyl ester aspartate was kindly given to us by Dr W. E. Brown of E. R. Squibb & Sons, Princeton, N.J., U.S.A. Miconazole nitrate was supplied by Dr H. van den Bossche, Janssen Pharmaceutica, Belgium.

RESULTS

Variation in miconazole sensitivity of cells and spheroplasts with phase of growth

Organisms harvested during exponential growth leaked K⁺ at a rate of 1 nmol min⁻¹ (mg dry wt)⁻¹ above that in the control in the presence of approximately 12 µg MN ml⁻¹; sensitivity decreased sharply as the culture entered the stationary phase, cells harvested after 36 h being very resistant (Table 1). Spheroplasts prepared from cells harvested after 18 and 36 h had the same sensitivity as exponentially growing cells (Table 1). As in the case of sensitivity to AME, stationary phase organisms were markedly more resistant than exponential phase organisms and the difference would appear to lie in changes in the cell wall. Organisms reached high levels of resistance (> 50 µg drug ml⁻¹) more rapidly for MN than for AME. Experiments were therefore carried out with 24 to 36 h cultures for tests on MN rather than the 5 to 8 d used for the AME studies.

Effect of oxygen saturation on development of MN resistance

Organisms were grown in a fermenter under controlled conditions; after growth was constant, the flow of medium was stopped while stirring and aeration were continued (Gale *et al.*, 1977). At intervals, organisms were harvested, washed and their MN sensitivity was determined. The increase in resistance was rapid and linear with time: a culture aerated at 1.4 l min⁻¹ had s.r.c. values of 4, 19 and 35 µg MN ml⁻¹ after 0, 24 and 48 h, respectively. The rate of increase decreased with increasing rates of aeration: after 24 h, the s.r.c. values were 22, 19, 15 and 11 µg MN ml⁻¹ for aeration rates of 0.45, 1.4, 2.7 and 4.0 l min⁻¹, respectively. These changes differ from those obtained in similar experiments on AME resistance where the rate of increase increased with time and with the rate of aeration (Gale *et al.*, 1977, Fig. 1).

Gale *et al.* (1977) also studied the effect of changing the O₂ saturation of the medium on the resistance to AME of starved organisms. The resistance of an aerated culture fell to less than 10 % of its initial value after a 60 min period in which the culture was gassed with O₂-free N₂ and the O₂ saturation fell from 97 to 50 %. On resuming aeration, the O₂ saturation

Table 1. *Effect of growth phase on the miconazole sensitivity of cells and spheroplasts of Candida albicans*

Candida albicans was grown in YNB medium (Gale, 1974) containing 1 % (w/v) glucose in a rotary incubator maintained at 37 °C. At the times indicated, samples of cultures were taken and suspensions were prepared for determination of the s.r.c. as described in Methods. Parallel samples were converted to spheroplasts as described by Gale *et al.* (1975) and the s.r.c. was determined in the presence of 0.8 M-sorbitol as stabilizer.

Time of incubation at 37 °C (h)	Standard release concentration of miconazole ($\mu\text{g ml}^{-1}$)		Culture density (mg dry wt cells ml^{-1})
	Intact cells	Spheroplasts	
6	11.5	—	0.2
12	13.4	—	1.42
18	12.5	11.5	1.63
24	30	—	1.64
30	48	—	1.64
36	>50	10	1.64

rose quickly to 95 % but resistance to AME increased only slowly, being restored to the initial value (before gassing with N_2) after a further 18 to 24 h aeration. Organisms starved and aerated at 2.7 l min^{-1} for 24 h had an s.r.c. value of $25 \mu\text{g MN ml}^{-1}$. This culture was then gassed for 60 min with O_2 -free N_2 ; the O_2 saturation fell to 52 %, but there was little effect on the MN resistance, the s.r.c. value decreasing to approx. $22 \mu\text{g MN ml}^{-1}$. Aeration at 2.7 l min^{-1} was then restored for 150 min and this was accompanied by a further decrease in resistance to an s.r.c. value of approx. $15 \mu\text{g MN ml}^{-1}$.

Treatment with SH-reactive agents

AME resistance of stationary phase *C. albicans* is markedly affected by treatment with SH-reactive agents: treatment with mercaptoethanol decreases resistance almost to that measured in exponentially growing organisms whereas *N*-ethylmaleimide or iodoacetamide increase resistance by 2- to 10-fold depending upon the time the culture has been in stationary phase (Gale *et al.*, 1975; 1978, Fig. 4). Similar tests have been carried out with NM using 26 h batch cultures of *C. albicans* (older cultures have an initial resistance too high for accurate estimation). In six experiments the resistance of the organisms on harvesting was $18.5 \pm 1.5 \mu\text{g MN ml}^{-1}$; after 1 h incubation with 0.2 M-2-mercaptoethanol it was $12.0 \pm 3.5 \mu\text{g MN ml}^{-1}$, and after 1 h incubation with 1 mM-*N*-ethylmaleimide it was $10.5 \pm 3.5 \mu\text{g MN ml}^{-1}$.

Treatment with enzymes

Gale *et al.* (1980) showed that AME resistance in 7 d batch cultures of *C. albicans* could be partially reduced by 24 h incubation at 37 °C and pH 6.5 with trypsin, lipase or chitinase and greatly reduced (to that of exponentially growing organisms) by digestion under the same conditions with (1 $\rightarrow$ 3)- β -D-glucanase. The effect of enzymes on MN resistance was tested in similar experiments carried out with 6 d batch cultures (s.r.c. > $50 \mu\text{g MN ml}^{-1}$). Neither trypsin nor lipase digestion had any significant effect, while chitinase, at a final concentration of $100 \mu\text{g protein (mg dry wt organisms)}^{-1}$, reduced the resistance to approx. $10 \mu\text{g MN ml}^{-1}$, i.e. that of exponentially growing organisms. Treatment with the Cytophaga L1 glucanase preparation gave very variable results suggesting (see Gale *et al.*, 1980) that a proportion of the organisms became sensitive to MN while the resistance of the remainder of the culture was unaffected.

DISCUSSION

Both AME and MN affect the membranes of *C. albicans* with the result that cells begin to leak K⁺ ions. The time course of the leakage is different: the effect with AME is time- and concentration-dependent whereas with MN the leakage is concentration-dependent but constant with time once established. In both cases stationary phase cultures are resistant and removal of the cell wall releases sensitive spheroplasts. That the changes leading to resistance are different for the two drugs is shown by: (i) the onset of resistance to MN progresses linearly and is decreased by high aeration whereas the onset of resistance to AME increases with time and is dependent on high aeration; (ii) reduction of the organisms with mercaptoethanol, or of the medium by gassing with N₂, markedly decreases AME resistance but has relatively little effect on MN resistance; (iii) treatment of organisms with *N*-ethylmaleimide greatly increases AME resistance of stationary phase cultures but decreases the MN resistance of those cultures; and (iv) quantitative aspects of the effects of treatment of organisms with enzymes differ for measurements of resistance towards the two drugs.

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