Problems in the laboratory assessment of antifungal activity

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Summary

The end points of tube dilution tests for minimal inhibitory concentrations of miconazole and flucytosine against *Candida albicans* were difficult to evaluate because partial inhibition was noted over a wide range of antifungal concentrations. This problem was not encountered with amphotericin B. Partial inhibition of *Candida* arose because of reductions in yeast growth rate and of cell yield. Different sizes of yeast inocula were differentially inhibited by the same concentration of antifungal agent.

An *in vitro* apparatus was described in which miconazole formulated as commercial creams, pessaries and medicated tampons for intravaginal application could be assessed for its inhibitory action *in vitro*.

Introduction

Laboratory tests for antifungal sensitivities are usually restricted to determinations of the minimal inhibitory concentration (MIC) of pure antifungal substance for a given fungal isolate. The relationship between MIC and *in vivo* effectiveness of a given compound is not always clear, and in any case the commercial formulations of an antifungal that are used clinically are not, as a rule, tested in the laboratory. Antifungal MIC determinations are known to be affected by such performance variables as the composition of the culture medium (Hoeprich and Finn, 1972), the presence or absence of serum proteins (Sreedhara Swamy, Sirsi and Ramonanda Rao, 1974) and the size of the test inoculum (Galgiani and Stevens, 1976). In this presentation attention is drawn to an additional difficulty of assessment of MIC presented by apparent partial inhibition of yeast cultures by flucytosine and miconazole, and a suggestion is given for a new approach to laboratory evaluation of commercial antifungal formulations *in vitro*.

Materials and methods

Three strains of *Candida albicans*, 73/004, NCPF 3153 and ATCC 28366 were used in the experiments. They were maintained on slopes of Sabouraud's

dextrose agar (SDA, Difco) and inocula were prepared as aqueous suspensions of yeasts from slopes of SDA incubated for 18 hr at 37°C. Yeast cell numbers were estimated by haemocytometer counts.

For experiments to determine MIC and effects of inoculum size on MIC, *C. albicans* was inoculated into 10-ml lots of YNB (yeast nitrogen base, Difco) plus 1% glucose in 16-mm diameter sterile test tubes, and incubated at 37°C. Three antifungals were used in the tests: amphotericin B complexed with buffered desoxycholate (Fungizone i.v., E.R. Squibb) and flucytosine were dissolved in sterile water, miconazole was dissolved in acetone. The antifungal solutions were diluted in YNB medium to concentrations appropriate for the experiments. For determination of *C. albicans* growth rates and cell yields in the presence of antifungals, the experimental conditions were similar except that culture volumes of 100 ml in 250-ml flasks were shaken at 160 r.p.m. and 37°C. Turbidity of cultures was determined spectrophotometrically at 550 nm against a water blank.

The apparatus for *in vitro* experiments with intravaginal formulations of antifungals is illustrated in Fig. 1. The apparatus was sterilized in an autoclave. A miconazole vaginal pessary, miconazole-coated tampon or 5 ml of miconazole vaginal cream was introduced in the 1.4-cm diameter dialysis sac, and 4-6 ml of phosphate buffered saline (PBS), 10% bovine serum albumin (Sigma) in PBS or vaginal washings in PBS obtained from routine gynaecology out-patients was added to the dialysis sac at zero time. Yeast nitrogen base medium (30 ml) inoculated to 10⁶ *C. albicans* ATCC 28366/ml was added aseptically to the chamber surrounding the dialysis sac. The apparatus was incubated at 37°C and samples of the *C. albicans* culture were removed aseptically at intervals for estimation of yeast numbers with a haemocytometer. Just before each sample was removed the culture was homogenized by aeration with a sterile Pasteur pipette and rubber bulb. A control apparatus contained a plain tampon within the dialysis sac. The rationale of the model was that miconazole within the cream, pessary, etc.

FIG. 1. Apparatus for *in vitro* assessment of intravaginal antifungal formulations. The antifungal cream, pessary or tampon was introduced in the dialysis sac together with buffered saline, protein solution or vaginal washings. A culture of *C. albicans* was placed in the space surrounding the dialysis sac.

would have to diffuse through the dialysis membrane, without being bound to proteins, in order to inhibit the *C. albicans* culture.

FIG. 2. Turbidity of a culture of *C. albicans* in tube dilution tests for antifungal sensitivities. (a) amphotericin B, (b) flucytosine, (c) miconazole. Two other strains tested produced similar curves.

Results

MIC determinations

When the turbidity of 3 strains of *C. albicans* cultures was measured 24 and 48 hr after inoculation at 10^6 /ml into media containing doubling dilutions of antifungals, different inhibitory effects were noted for amphotericin B, flucytosine and miconazole (Fig. 2). As the concentration of amphotericin B increased, the culture turbidity remained essentially the same as that of an amphotericin-free control until at inhibitory concentrations of amphotericin B there was an abrupt fall in culture turbidity (Fig. 2a). The change in culture turbidity with amphotericin B concentration occurred over 3 antifungal dilutions when turbidities were measured after 24 hr's incubation, and over 2 dilutions after 48 hr. By contrast, with flucytosine, the culture turbidity fell steadily from maximum (no inhibition) to minimum (full inhibition) over at least 4 tube dilutions (Fig. 2b); that is, partial inhibition was noted over a 16-fold range of flucytosine concentration. For miconazole, partially inhibitory effects were observed over a 128-fold range of concentration for all 3 strains (Fig. 2c). The wide ranges of antifungal concentration over which partial inhibition was seen for flucytosine and miconazole rendered naked-eye determination of MIC highly liable to error.

In experiments where the inhibitory effects of the 3 test antifungals were assessed in terms of growth rate and cell yield of *C. albicans* ATCC 28366, it was observed that the effects of amphotericin B ranged from nil at 0.02 mg/l to fungicidal at 2.0 mg/l (Table 1). Partial inhibition by this agent was achieved at 0.2 mg/l. Flucytosine at concentrations up to 0.2 mg/l caused a reduction in maximum growth rate of the cultures but did not affect cell yield, while miconazole at concentrations from 0.2 to 20.0 mg/l reduced both growth rate and cell yield in proportion to concentration.

TABLE 1. Growth rates and cell yields of *C. albicans* ATCC 28366 in the presence of antifungals (determined from growth curves of *C. albicans*)

Concentration (mg/l)	Amphotericin B		Flucytosine		Miconazole	
	Maximum growth rate/hr	Relative cell yield*	Maximum growth rate/hr	Relative cell yield*	Maximum Growth rate/hr	Relative cell yield*
0.00	0.82	1.8	0.82	1.7	0.79	1.8
0.02	0.82	1.8	0.35	1.7		
0.02	0.52	0.5	0.10	1.7	0.71	1.8
2.00	0.0	—†	0.06	—‡	0.38	1.6
20.00					0.09	0.5

* Calculated as log optical density (OD)₅₅₀ at stationary phase minus log OD₅₅₀ at zero time.

† Turbidity fell throughout experiment, indicating cell death.

‡ No clear-cut stationary phase was attained within the time course of the experiment.

TABLE 2. Variation of *C. albicans* yield with inoculum size – data show OD₅₅₀ after 48 hr's incubation

Yeasts/ml (initial)	Control (no antifungal)	Amphotericin B (0.5 mg/l)	Flucytosine (0.2 mg/l)	Miconazole (0.5 mg/l)
10 ⁷	1.55	1.55	1.29	1.23
10 ⁶	1.53	1.54	0.76	0.66
10 ⁵	1.54	0.04	0.01	0.07
10 ⁴	1.55	0.04	0.02	0.04

Variation of the inoculum size in tube MIC determinations led to different gross inhibitory effects on *C. albicans* for the same antifungal concentration (Table 2). Amphotericin B at 0.5 mg/l was fully inhibitory to inocula of 10⁵ yeasts/ml and fully non-inhibitory to inocula of 10⁶/ml or greater, whereas with flucytosine and miconazole, intermediate turbidity figures between those for the smallest and the largest inocula indicated inoculum-dependent partially inhibitory effects.

Inhibition of *C. albicans* in the vaginal antifungal model

The data in Table 3 show that all 3 miconazole vaginal formulations tested in the apparatus shown in Fig. 1 effectively inhibited the growth of *C. albicans* in the fluid surrounding the dialysis sac. The effects of the formulations were the same regardless of the presence or absence within the sac of

albumin solution or vaginal washings. In experiments where the growth of *C. albicans* around the dialysis sac was monitored with time, the miconazole cream formulation was immediately fungistatic whereas the miconazole pessary and coated tampon formulations caused fungistasis after one yeast generation.

Discussion

This study has highlighted some of the difficulties that may confound precise determination of *in vitro* MIC for flucytosine or miconazole. A reasonably clear-cut MIC end point was achieved for amphotericin B, but in flucytosine and miconazole sensitivity tests the series of tubes contain cultures of steadily decreasing turbidity, which results in subjective, and therefore variable end-point determinations. For all 3 antifungals the inhibitory effects

TABLE 3. Total cell counts of *C. albicans* before and after incubation at 37°C for 24 hr in culture fluid surrounding dialysis tube of model vagina. Essentially the same results were obtained regardless of the presence within the tube of 10% albumin solution or vaginal washings, so the data from all tests are pooled in the table

Preparation tested in dialysis tube	Log ₁₀ yeasts/ml, mean (± s.e. mean)		Number of replicates
	per ml at zero time	per ml at 24 hr	
Plain tampon	5.9 (0.1)	7.5 (0.0)	4
Miconazole absorbent pessary	5.9 (0.2)	6.0 (0.2)	5
Miconazole cream	6.0 (0.0)	5.9 (0.1)	4
Miconazole pessary	6.0 (0.0)	6.3 (0.0)	3

varied enormously with the size of the test inoculum. Galgiani and Stevens (1976) recommended the routine use of turbidity measurements in antifungal MIC assays, since a 50% turbidity reduction figure proved to be more consistent and inoculum-independent than MIC. In view of the large range of concentration of miconazole and flucytosine over which partial inhibition of *C. albicans* was seen in the present study, it is clear that some objective, turbidity-based end-point is likely to be more reproducible from laboratory to laboratory; however, further experience with sensitivity tests will be required before such end-points may be related even circumstantially to the clinical efficacy of antifungal compounds. Inter-laboratory co-operation should, in time, lead to the introduction of a standard sensitivity test protocol for antifungal agents.

The experiments with the vaginal model apparatus (Fig. 1, Table 3) provide a preliminary indication that commercial formulations of antifungal agents may be as amenable to laboratory investigation as pure antifungal compounds. The model has demonstrated that miconazole, in 3 different intravaginal formulations, is rapidly released, without impediment by protein binding, in concentrations sufficient

to inhibit the growth of a surrounding *C. albicans* culture: this *in vitro* finding broadly correlates with *in vivo* results obtained in clinical trials of miconazole (Odds, 1977).

Acknowledgments

The author wishes to thank Janssen Pharmaceutical Ltd for the gift of miconazole nitrate and its commercial formulations, Roche Products Ltd for the gift of flucytosine, and L. A. Fraser and A. Abbott for their skilled technical assistance.

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