SUSCEPTIBILITY OF FUNGI IN MOUTHRINSE SPECIMENS FROM PATIENTS WITH HAEMATOLOGICAL MALIGNANCIES

A. A. I. KOSTIALA AND I. KOSTIALA

*Department of Bacteriology and Immunology, University of Helsinki,
00290 Helsinki 29, Finland*

SUMMARY. Fungi isolated from mouthrinse specimens during episodes of acute pseudomembranous fungal stomatitis and deep-seated mycoses in patients with haematological malignancies were tested for susceptibility to seven antifungal agents. Topical treatment of stomatitis with clotrimazole or chlorhexidine did not induce any change in the susceptibility of oral *Candida albicans*. Treatment of deeper mycoses with 5-fluorocytosine, however, resulted in a significant increase in oral strains resistant to this agent. Of *C. albicans* strains isolated, 7% were resistant to 5-fluorocytosine $> 32 \mu\text{g/ml}$. One patient died of disseminated mycosis during treatment with this drug; the resistant *C. albicans* was isolated from the mouth, liver, spleen and kidneys. Strains of *Torulopsis glabrata* and *C. krusei* resistant to 5-fluorocytosine were also found in some patients. Organisms resistant to 5-fluorocytosine were generally sensitive to polyenes and imidazoles.

INTRODUCTION

Opportunistic infections are a major problem in immunocompromised patients and fungal infections are diagnosed with increasing frequency (Hotchi, Okada and Nasu, 1980; Stahel *et al.*, 1982; Kostiala, 1984). There are relatively few antifungal drugs and some are suitable for topical treatment only. Clotrimazole lozenges have been found to be effective in acute pseudomembranous fungal stomatitis (Speller, 1979; Kostiala *et al.*, 1982a). Other agents, such as 5-fluorocytosine, may be administered by the oral and intravenous routes with relatively few adverse effects. However, both primary and secondary resistance to this agent has been reported (Hoeprich *et al.*, 1974; Speller, 1979). Sensitivity testing of pathogenic fungi may be done by various methods; commercial preparations of disks containing antifungal agents are available for routine disk diffusion tests (Casals, 1979).

We have tested the sensitivity *in vitro* of fungi isolated from mouthrinse specimens during episodes of stomatitis and deep-seated fungal infection in patients with haematological malignancies. Stomatitis was treated with topical clotrimazole or chlorhexidine, whereas oral or intravenous 5-fluorocytosine was used against deeper infections. Susceptibility of fungi to several antifungal agents was tested by the disk diffusion and broth dilution methods.

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MATERIALS AND METHODS

Patients and organisms. Fungi were isolated from mouthrinse specimens of 53 patients with haematological malignancies in the haematology ward of the University Central Hospital, Helsinki from December 1979 to January 1981. The clinical details have been described elsewhere (Kostiala *et al.*, 1982a). Patients with stomatitis were treated topically with either 10-mg clotrimazole lozenges or 0.2% chlorhexidine gluconate mouthrinse five times daily for 14 days. Indications for oral or intravenous 5-fluorocytosine treatment included severe fungal stomatitis, verified or suspected fungal oesophagitis, enterocolitis or abscess, or suspicion of fungal septicaemia. A total of 20 patients was treated with oral 5-fluorocytosine (1.5–2.5 g four times daily) or intravenous 5-fluorocytosine (2.5 g four times daily); these patients were treated for a mean of 18 days (range 5–45 days). Another 32 strains of fungi from mouthrinse specimens of 40 control patients from the same ward were studied; these patients exhibited no clinical evidence of fungal infection.

Mouthrinse samples were taken in the morning before breakfast and brushing of teeth. Subjects rinsed the mouth for one min with 10 ml of phosphate buffered saline, pH 7.4. Specimens were cultured on seven media routinely used in the Mycological Laboratory, Department of Bacteriology and Immunology, University of Helsinki. Determination of species was done as described earlier (Kahanpää, 1972). For quantitative culture, 0.1 ml of specimens were plated on Sabouraud dextrose agar (Oxoid) plates containing penicillin 120 µg/ml and streptomycin 250 µg/ml and the number of cfu/ml determined (Kostiala *et al.*, 1982a and b). Disk diffusion tests were performed immediately after isolation of fungi. For the determination of minimal inhibitory concentrations the organisms were maintained in Sabouraud dextrose agar tubes at 4°C and subcultured on Yeast Nitrogen Base (YNB, Difco) agar plates when required for testing.

Antifungal agents. Stock solutions of antifungal drugs at concentrations of 12.8 mg/ml were prepared as recommended by Holt (1975); 5-fluorocytosine (Ancotil®, Hoffmann-LaRoche et Co., Basel, Switzerland) was diluted in 0.01 M phosphate buffer, pH 7.0, amphotericin B (Fungizone®, E.R. Squibb and Sons, London) was dissolved in distilled water, and clotrimazole (Canesten®, Bayer AG, Leverkusen, Germany) and ketoconazole (Nizoral®, Janssen Pharmaceuticals, Beerse, Belgium) were dissolved in dimethylformamide (Merck, Darmstadt, Germany). The stock solutions were stored at –20°C.

Disk diffusion method. The disk diffusion test described by Casals (1979) was used. YNB plates were seeded with suspensions of yeasts (*c.* 10⁵ cfu/ml) in 0.01 M phosphate buffer, pH 7.0, and Neo-Sensitabs tablets (A/S Rosco-Denmark, Taastrup, Denmark) were applied to the surface of the plates. The tablets contained the following drugs: 5-fluorocytosine (10 µg), amphotericin B (20 I.U.), nystatin (50 µg), clotrimazole (10 µg), miconazole (10 µg), ketoconazole (10 µg) and econazole (10 µg). The plates were incubated for 24 h at 37°C (28°C in the case of *Geotrichum candidum*) and the zones of inhibition measured up to colonies of normal size. The results were recorded as sensitive (S), intermediate (I) or resistant (R) according to the criteria recommended by the manufacturer.

Broth dilution method. Minimal inhibitory concentrations (MICs) were determined by the method described for 5-fluorocytosine by Shadomy (1969), with minor modifications. Twofold serial dilutions of drugs (range 64–0.063 µg/ml) were prepared in 1-ml volumes of YNB in 0.01 M phosphate buffer, pH 7.0, supplemented with L-asparagine and dextrose as described by Holt (1975). The tubes were seeded with 1-ml volumes of suspensions of the fungus in 0.01 M phosphate buffer, pH 7.0, containing Triton X-100 0.1% and phenol red 0.15%. The inoculum density was *c.* 10⁵ cfu/ml. Control tubes containing YNB broth without drug and YNB broth with dimethylformamide 0.5% v/v were also seeded with the fungus. The inoculum purity and density were checked by plating on Sabouraud dextrose agar plates. The tubes were incubated for 24 h at 37°C (28°C for *G. candidum*). The MIC was defined as the lowest concentration of drug that inhibited visible growth.

RESULTS

Susceptibility of oral Candida albicans from infected patients

Topical treatment was used as primary medication in 85 episodes of fungal stomatitis in 53 patients. Susceptibilities of oral *C. albicans* from patients who completed the 14-day course of treatment and from whom the organism was still isolated from mouthrinse specimens are presented in table I. At the beginning of treatment, most of the isolates were sensitive to all the antifungal drugs tested and little change in susceptibility occurred during topical treatment.

Twenty-one episodes of fungal infection in 20 patients were treated with oral or intravenous 5-fluorocytosine for a mean of 18 days (range 5–45 days). Whereas all oral isolates of *C. albicans* were sensitive to 5-fluorocytosine at the beginning of treatment, 24% of isolates were resistant at the end of treatment (table I). These isolates were derived from five episodes of fungal infection that occurred in four patients.

Comparison of the susceptibility of oral C. albicans in infected and non-infected patients

Twenty-one strains of *C. albicans* were isolated from 40 control patients with haematological malignancies, but without fungal infection. There was no essential difference between the results obtained with strains from infected and control patients, apart from an increased number of *C. albicans* isolates resistant to 5-fluorocytosine among 83 isolates from patients with fungal infection.

TABLE I

Susceptibility of oral C. albicans from patients with episodes of fungal stomatitis treated with topical clotrimazole or chlorhexidine and with episodes of deep-seated fungal infection treated with 5-fluorocytosine

Antifungal drug	Susceptibility category	Percentage in each susceptibility category before and after treatment with					
		Clotrimazole* (n = 28)		Chlorhexidine† (n = 23)		5-fluorocytosine‡ (n = 21)	
		before	after	before	after	before	after
5-fluorocytosine	S	100	96	100	100	100	76
	I	0	4	0	0	0	0
	R	0	0	0	0	0	24
Amphotericin B	S	93	96	96	100	95	95
	I	7	4	4	0	5	5
Nystatin	S	100	100	100	100	100	100
Clotrimazole	S	93	93	96	100	100	95
	I	7	7	4	0	0	5
Miconazole	S	93	96	91	87	95	90
	I	0	4	0	4	5	10
	R	7	0	9	9	0	0
Econazole	S	75	86	83	83	90	86
	I	7	0	4	0	5	5
	R	18	14	13	17	5	9

S = fully sensitive; I = intermediate sensitivity; R = resistant.

* Clotrimazole lozenges for 14 days.

† Chlorhexidine gluconate mouthrinse for 14 days.

‡ Oral or intravenous 5-fluorocytosine for a mean of 18 days.

Overall, 7% of *C. albicans* strains tested were resistant to 5-fluorocytosine (MIC > 32 µg/ml in each case); 4% showed intermediate sensitivity to amphotericin B; all were sensitive to nystatin; 10% exhibited intermediate sensitivity to clotrimazole; 11% were resistant, or of intermediate sensitivity, to miconazole; and 18% were resistant, or of intermediate sensitivity, to econazole. All but one of the seven 5-fluorocytosine-resistant strains of *C. albicans* were fully sensitive to all other antifungal agents tested. The single exception showed reduced susceptibility to amphotericin B, miconazole and econazole.

Susceptibility of oral fungi other than C. albicans

Various fungi other than *C. albicans* were isolated during episodes of fungal infection (Kostiala *et al.*, 1982a and b). The susceptibility patterns of some are summarised in table II. Resistance to 5-fluorocytosine was found in two strains of *Torulopsis glabrata* (MIC > 32 µg/ml) and two strains of *C. krusei* (MIC 16 µg/ml). One *T. glabrata* strain and both *C. krusei* strains exhibited reduced sensitivity to amphotericin B (MIC 2 or 4 µg/ml). One *C. krusei* strain was fully susceptible only to clotrimazole of the agents tested. With the exception of *G. candidum*, most isolates were sensitive to the imidazoles tested, except econazole, to which many strains were resistant or of intermediate sensitivity.

Similar sensitivity patterns were seen in six *G. candidum* and two *C. parapsilosis* strains obtained from 40 control patients without stomatitis. Additionally, three control patients harboured *T. glabrata*; one strain was resistant to 5-fluorocytosine and showed intermediate sensitivity to clotrimazole (data not shown).

Origin of 5-fluorocytosine-resistant yeasts

C. albicans strains resistant to 5-fluorocytosine were isolated from seven patients with fungal infection. In four patients the resistant organism appeared during treatment with 5-fluorocytosine (table I). One of these patients, suffering from acute myeloid leukaemia, died of disseminated mycosis and the resistant organism was isolated from the mouth, liver, spleen and kidneys. Two of the seven patients had

TABLE II
Susceptibility to various antifungal agents of oral fungi other than C. albicans

Species	Number of strains tested	Number of strains (MIC range, µg/ml) showing resistance or reduced susceptibility to						
		5-fluoro-cytosine	ampho-tericin B	clotrim-azole	ketocon-azole	mico-nazole	econ-azole	ny-statin
<i>Geotrichum candidum</i>	10	1 (0.5-2)*	3 (1-2)	6 (2-16)	6 (1-8)	4	7	0
<i>Torulopsis glabrata</i>	4	2 (0.125->32)	3 (0.03-4)	2 (0.03-16)	0 (<0.03-2)	0	1	0
<i>Saccharomyces cerevisiae</i>	3	0 (0.03-2)	1 (1-2)	1 (1-8)	0 (0.06-0.5)	0	0	0
<i>Candida krusei</i>	2	2 (16)	2 (2-4)	0 (0.25)	1 (1)	1	2	1
<i>Candida parapsilosis</i>	2	0 (1-2)	1 (1-4)	0 (0.06-0.5)	0 (0.06-1)	0	2	0
<i>Candida guilliermondi</i>	1	0 (0.125)	0 (1)	0 (<0.03)	0 (<0.03)	0	1	0
<i>Hansenula anomala</i>	1	0 (0.25)	0 (1)	0 (0.06)	0 (<0.03)	1	1	1

* The figures in brackets indicate the MIC range (µg/ml) in broth dilution titrations.

received 5-fluorocytosine one year earlier; one patient harbouring a resistant strain had no known contact with the drug.

One of the two patients from whom 5-fluorocytosine-resistant *T. glabrata* was isolated was treated with 5-fluorocytosine for fungal enterocolitis; resistant *T. glabrata* was isolated from the mouth during therapy. No patient had more than one 5-fluorocytosine-resistant species of yeast.

DISCUSSION

In the present investigation, the susceptibility of fungi isolated from mouthrinse specimens from patients with haematological malignancies was studied. Many of these patients had fungal stomatitis, a condition in which abundant growth ($\geq 10^3$ cfu/ml of mouthrinse) of fungi occurs (Kostiala *et al.*, 1982a and b). By use of mouthrinse specimens, a specimen for mycological culture which probably reflects the fungal flora harboured by the patient can be obtained easily and conveniently. Topical treatment of stomatitis with clotrimazole or chlorhexidine did not induce any change in the susceptibility of oral *C. albicans* (table I). Similar results have been obtained with topical clotrimazole in vaginal candidosis (Milne, 1974) and with chlorhexidine in oral candidosis (Cannell, 1981). However, when 5-fluorocytosine was used in the treatment of severe fungal infection, strains resistant to this drug appeared in the oral cavity (table I).

The present results on the susceptibility of *C. albicans* are in general agreement with those reported by investigators from other parts of the world (Hamilton-Miller, 1972; Auger, Dumas and Joly, 1979; Defever *et al.*, 1982; Dermoumi, 1982; Stiller *et al.*, 1982). Thus 7–43% of *C. albicans* strains are resistant to 5-fluorocytosine, but reduced sensitivity to amphotericin B is rare. Of the imidazoles investigated in the present study, intermediate susceptibility to clotrimazole occurred in c. 10% of the strains and reduced susceptibility or resistance to econazole was more common. Although strains resistant to imidazoles *in vitro* are being encountered (Speller, 1979), the clinical significance of this is not known.

As well as *C. albicans*, strains of *C. krusei* and *T. glabrata* resistant to 5-fluorocytosine were found in the mouths of haematological patients. The clinical importance of 5-fluorocytosine resistance is indicated by the fact that one patient died of disseminated mycosis while receiving 5-fluorocytosine; resistant *C. albicans* was isolated from the mouth and also from liver, spleen and kidneys *post mortem*. This agrees with the results of Stiller *et al.* (1983) who showed that *in-vitro* testing was able to predict the *in-vivo* response to treatment with 5-fluorocytosine in a mouse model of systemic candidosis. It has been suggested that 5-fluorocytosine-resistant variants may be selected from a pre-existing heterogeneous population by exposure to the drug *in vitro*, and this may also be the mechanism of development of resistance *in vivo* (Whelan *et al.*, 1981; Defever *et al.*, 1982). As pointed out by Whelan *et al.* (1981), the use of 5-fluorocytosine in treating infections due to strains heterogeneous for resistance seems inadvisable because of the possibility of selection for the resistant variants. A screening procedure for the detection of such variants was suggested by Whelan *et al.* (1981); however, this test remains to be evaluated in practice. In any case, laboratory control of therapy by sensitivity tests is indicated during 5-fluorocytosine therapy (Speller, 1979). Fortunately, the present results, and those of others (Hamilton-

Miller, 1972; Hoeprich *et al.*, 1974) indicate that 5-fluorocytosine resistant strains retain susceptibility to polyenes and imidazoles.

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