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Influence of antifungal pre-treatment in preparing test mycelium on MIC values of several antifungal agents against *Aspergillus niger*

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Antifungal activity of two imidazoles (miconazole and ketoconazole) and one polyene (amphotericin B) was evaluated using an automatic growth analysis system. Spores of *Aspergillus niger* were inoculated on the polylysine-coated glass bottom of a culture vessel. A colony formed in liquid medium was exposed to an antifungal agent and subsequently washed. Based on the dynamic growth rate of a test hypha selected from the colony in response to the antifungal agent, the minimum inhibitory concentration (MIC) was evaluated. The influence of time of reading (1, 2 and 3 h after washing) on the MIC determined was investigated. MICs for test hyphae subjected to antifungal pre-treatment were compared with those for hyphae without pre-treatment. Hyphae pre-treated with an antifungal agent for 1 h were found to become adapted and tolerant to that antifungal agent. Hyphae exposed and adapted to an imidazole obtained tolerance to amphotericin B as well as to the other imidazole.

Key Words——adaptation; antifungal pre-treatment; *Aspergillus niger;* minimum inhibitory concentration (MIC).

Cellular adaptation of fungi to antifungal agents is a continuing problem in the fields of medical science, agriculture and environmental sanitation. The introduction of imidazoles to clinical practice in therapy of mycoses often resulted in failure because of the occurrence of resistant strains (Holt and Azmi, 1978; Ryley et al., 1984). It was reported that *Penicillium digitatum* and *P. italicum* could develop resistance or tolerance to thiabendazole and potassium sorbate used in control of postharvest mold spoilage of citrus fruits (Harding, 1972; Schroeder and Bullerman, 1985), and several fungal strains isolated from the molded walls could also develop resistance to thiabendazole (Takatori et al., 1981). However, because of the difficulties in analysis of the changes taking place in mycelia (Lenhart, 1973), adaptation studies using filamentous fungi at the mycelial and/or hyphal level have been rare, and restricted to the possibility of development of tolerant strains by repeated and long-term exposure (at least for several days) to subinhibitory levels of these antifungal agents (Takatori et al., 1981; Schroeder and Bullerman, 1985; Lenhart and Merkunova, 1989).

Previously, a system for the evaluation of dynamic growth of a single hypha was developed and its applicability to the evaluation of antifungal activity of several antifungal agents was demonstrated (Yamada et al., 1992; Oh et al., 1992). This system was applied to the study of adaptation to short-term salt stress and several antifungal agents at the single hypha level by assessing the response of the same hypha to repeated exposure to the same stress (Park et al., 1993, 1994). In the present study, the minimum inhibitory concentrations (MICs) of several antifungal agents against *Aspergillus niger* were determined on the basis of the dynamic growth rate of a single hypha in liquid medium, and the effects of short-term exposure (for 1 h) to antifungal agents in preparing test mycelium on MICs were investigated. It was confirmed that hyphae pretreated with antifungal agents became tolerant to those antifungal agents.

Materials and Methods

Organism and antifungal agents *Aspergillus niger* IFO 6661 was maintained at 4°C on potato-dextrose agar slants (PDA, Difco). The fungus was precultured on PDA slants at 28°C for 7 d prior to use. Amphotericin B and miconazole were purchased from Sigma and ketoconazole from Biomol Research Laboratories. Stock solutions were prepared at 10 mg/ml in dimethyl sulphoxide (DMSO) immediately prior to use. For MIC de-

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termination, twofold dilutions of the antifungal agents were prepared in potato-dextrose broth (PDB, Difco) at concentrations ranging from 0.1 to 100 μg/ml. Final concentrations of DMSO in the test drug preparations were less than 0.25% (v/v) (Park et al., 1994).

System for measuring dynamic hyphal growth in liquid medium The system described in a previous paper (Park et al., 1994) was used in measuring dynamic hyphal growth in liquid medium. The glass bottom plate of the culture vessel was coated with poly-L-lysine (poly-L-lysine hydrobromide MW 70,000-150,000, Sigma). In the image seen through a microscope, an appropriate hypha was selected and its growth rate was calculated from the distance moved by its apical position during the measuring interval (Yamada et al., 1992; Oh et al., 1992).

Assay procedure of antifungal activity using automatic growth analysing system *Aspergillus niger* spores were harvested with sterile saline containing 0.05% (v/v) Tween 80 from 7-d-old cultures grown on PDA slants at 28°C. After vigorous agitation, the suspension was filtered through glass wool and the spores were washed three times with sterile distilled water. The spores were resuspended and adjusted to 2×10^8 spores/ml in sterile distilled water. One microlitre of the spore suspension was placed onto the poly-L-lysine-coated glass plate of culture vessel and left to stand for 30 min. The suspension was removed and the glass plate was rinsed with 10 ml of sterile distilled water. The adhesion of spores (about 10^3 spores) on the glass plate was confirmed by microscopic observation. Adhered spores did not move during the introduction of medium or sterile distilled water. One millilitre of PDB was poured into the culture vessel and incubated for 24 h at 28°C to form a colony with a diameter of about 5 mm. The culture vessel containing test mycelium was mounted on the microscope stage, and the medium was replaced by 1 ml of PDB and left to stand for 30 min at 28°C. An appropriate hypha that was growing horizontally while adhering to the glass plate was selected as a test hypha. The apical position of the hypha was automatically traced during the following steps (Fig. 1):

1. The growth rate of the hypha was measured for 30 min in the PDB containing no antifungal agents and confirmed to be at a steady level (pre-exposure).
2. The medium was replaced by the PDB containing a given concentration of antifungal agent, and the hypha was exposed to the antifungal agent for 60 min (exposure).
3. The PDB containing the antifungal agent was removed and the hypha was washed with PDB containing no antifungal agent (post-exposure).

During these three steps, the growth rate was recorded continuously. The MIC of the antifungal agent was determined as the minimum concentration necessary to cause complete inhibition of hyphal growth, i.e., the concentration at which τ_{OFF} (lag time from the termination of exposure to an antifungal agent until the growth rate begins to change in response to washing) was longer than the time of MIC reading.

MIC determination by dry weight measurement MICs of antifungal agents were evaluated from the inhibition effect on the dry weight increase of *A. niger* in a thin layer of liquid medium using the method of Cohen (1973). Serial two-fold dilutions of each antifungal agent were prepared in PDB. The dilution scheme for each antifungal agent was within 0.78-200 μg/ml in a total volume of 20 ml per Petri dish. Each Petri dish was inoculated with 1 ml mycelial suspension, which was then incubated for 72 h at 28°C. Dry weight determinations were made on mycelia harvested from 20-ml culture samples. The mycelial mat was washed with 20 ml of distilled water and dried overnight at 105°C (Fletcher and Trinci, 1981).

Results

Influence of the time of reading on MIC determination As shown in Fig. 1, PDB containing antifungal agent was introduced after 60 min of pre-exposure, the mycelium was washed 60 min later, and MIC was determined in the post-exposure period. The parameter τ_{OFF} for 0.2 and 0.39 μg/ml miconazole was about 45 and 105 min, respectively, and that for 1.56 μg/ml was longer than 3 h. Therefore, if the time of MIC reading were 1 h after washing, the MIC of miconazole would be determined as 0.39 μg/ml, because τ_{OFF} for this concentration was longer than the time of MIC reading. If the time of reading were 2 h, however, MIC would be determined as

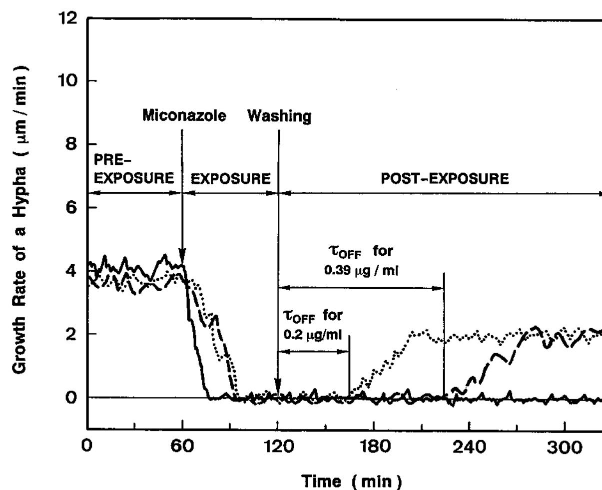

Fig. 1. Dynamic response curves of *A. niger* to various concentrations of miconazole: τ_{OFF}, lag time from the termination of exposure to antifungal agent until the growth rate begins to change in response to washing. At the arrow labeled washing, PDB containing miconazole at concentrations of (μg/ml) 0.2 (···), 0.39 (- - -), 1.56 (—) was replaced by fresh PDB. MIC was determined as the concentration at which τ_{OFF} was longer than the time of MIC reading. Experiments were done in triplicate for each concentration. Each line shows a representative result for that concentration.

Table 1. Comparison of the times of MIC reading of several antifungal agents against *A. niger* in the present system.

Antifungal agent	MIC (μg/ml) determined by			
	Hyphal growth method[a, b]			Dry weight method[b]
	1 h	2 h	3 h	
Miconazole	0.39	1.56	1.56	6.25
Ketoconazole	3.13	25	25	100
Amphotericin B	1.56	1.56	1.56	12.5

a) MIC reading was carried out for 1, 2, and 3 h after the washing with PDB containing no antifungal agent.
b) Experiments were done in triplicate.

1.56 μg/ml; and if 3 h, MIC would also be 1.56 μg/ml. MICs of miconazole, ketoconazole and amphotericin B determined with different reading times (1, 2 and 3 h) are summarized in Table 1. For miconazole and ketoconazole, MICs determined in 2 h of reading were the same those determined in 3 h as 1.56 and 25 μg/ml, respectively, and higher than those determined in 1 h as 0.39 and 3.13 μg/ml, respectively. For amphotericin B, different times of reading had no influence on the MIC of 1.56 μg/ml. In subsequent experiments, 2 h from washing was chosen as the time of MIC reading.

The MICs of these antifungal agents against *A. niger* obtained by the present method were compared with those determined by dry weight method, one of the conventional methods, as also shown in Table 1. The MICs determined by dry weight method were 6.29, 100 and 12.5 μg/ml for miconazole, ketoconazole and amphotericin B, respectively, four times higher than those determined by the present method (1.56, 25 and 1.56 μg/ml, respectively).

Influence of antifungal pre-treatment in preparing test mycelium on MIC determination The MICs of antifungal agents for hyphae cultured in medium containing no antifungal agent were compared with those from the hyphae exposed to a subinhibitory concentration of an antifungal agent. Figure 2 shows the effects of 0.01, 1.0 and 0.1 μg/ml of miconazole, ketoconazole and amphotericin B, respectively, on *A. niger* hyphal growth. In

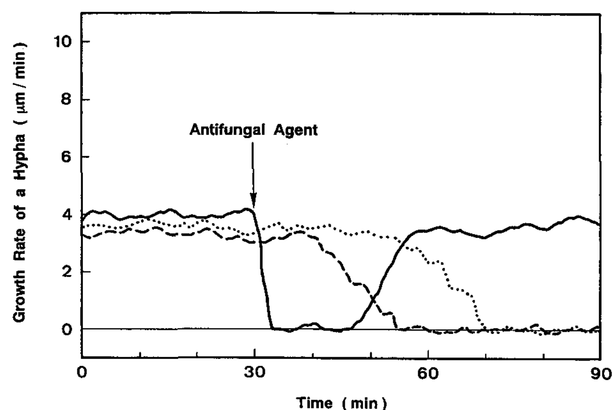

Fig. 2. Effect of antifungal agents on *A. niger* hyphal growth. At the arrow, PDB medium was replaced by PDB containing each antifungal agent. Concentrations (μg/ml) were: amphotericin B, 0.1 (—); miconazole, 0.01 (···); ketoconazole, 1.0 (- - -). Experiments were done in triplicate. The data are representative results for each antifungal agent.

the case of miconazole and ketoconazole, hyphal growth decreased gradually and ceased about 25 and 40 min, respectively, after introduction of the drug, while in the case of amphotericin B, it ceased immediately and resumed about 20 min later. In subsequent experiments, test hyphae were prepared by pre-treatment with these subinhibitory concentrations of the antifungal agents.

Table 2 shows the MICs of antifungal agents against *A. niger* hypha pre-treated for 1 h with each antifungal agent. The MICs for hyphae pre-treated with miconazol and ketoconazole were 6.25 and 100 μg/ml, respectively, four times higher than those for the hypha with no antifungal pre-treatment. For amphotericin B, the MIC (3.13 μg/ml) was two times higher after pre-treatment. The MICs of miconazol and ketoconazole for hyphae pre-treated with ketoconazole and miconazole, respectively, were each four times higher, and ketoconazole pre-treatment increased the MIC of amphotericin B two times. However, amphotericin B pre-treatment had no influence on the MIC of the two imidazole antifungal agents.

Table 2. Comparison of MICs of several antifungal agents against *A. niger* pre-treated for 1 h with PDB containing antifungal agents.

Antifungal agent	MIC (μg/ml)[a, c]			
	No antifungal agent[b]	Miconazole[b]	Ketoconazole[b]	Amphotericin B[b]
Miconazole	1.56	6.25	6.25	1.56
Ketoconazole	25	100	100	25
Amphotericin B	1.56	1.56	3.13	3.13

a) MIC was read for 2 h after the washing with PDB containing no antifungal agent.
b) After 24 h of incubation in PDB containing no antifungal agent, *A. niger* hypha was pre-treated for 1 h with PDB containing either miconazole (0.01 μg/ml), ketoconazole (1.0 μg/ml), amphotericin B (0.1 μg/ml) or no antifungal agent.
c) Experiments were done in triplicate.

Discussion

The imidazoles (miconazole and ketoconazole) and polyene (amphotericin B) used in this study differ in their antifungal action mechanism, the former two being fungistatic and the latter fungicidal (Van den Bossche, 1978; Yamaguchi and Hiratani, 1984; Hiratani and Yamaguchi, 1985; Bolard, 1986). As shown in Table 1, the MICs of the two imidazoles determined in 2 h of reading were higher than those determined in 1 h of reading. In contrast, the MIC of amphotericin B did not vary with the time of reading. This result appears to be caused by the difference in action mechanism between these antifungal agents.

As shown in Table 2, pre-treatment for 1 h with an antifungal agent increased the MIC of that agent two or four times over that for hyphae without antifungal pretreatment. This suggested that *A. niger* adapted to each antifungal agent during the pre-treatment. This in turn suggests that, in the case of conventional methods, the response of test fungi could change during the evaluation of antifungal activity, since these methods generally require several days to determine MIC.

Aspergillus niger adapted to one antifungal agent during pre-treatment obtained tolerance to the other antifungal agents, except in the case of amphotericin B pretreatment and miconazole pre-treatment against amphotericin B. These phenomena were regarded as cross-adaptation. Cross-adaptation was most clearly observed in an imidazole-adapted *A. niger* against amphotericin B as well as the other imidazole. And these results agree with the study that ketoconazole and amphotericin B have antagonistic effects in murine aspergillosis (Schaffner and Frick, 1985).

In the present study, the pre-treatment (1 h) was of short duration compared with previous studies of fungal adaptation to antifungal agents (Takatori et al., 1981; Schroeder and Bullerman, 1985; Lenhart and Merkunova, 1989). However, these short-term stresses of antifungal agents were found to be enough to influence MIC determination. The present study should serve as a basis for more thorough examination of the topic.

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