Mycoscience 36: 341–344, 1995

Studies on the synergistic effect of amphotericin B and 5-fluorocytosine on the growth rate of single hyphae of *Aspergillus fumigatus* by a biocell-tracer system

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Accepted for publication 3 July 1995

The biocell-tracer system is a microscopical system to measure the growth rate of a single fungal hypha. The synergistic effect of amphotericin B (AMPH) and 5-fluorocytosine (5-FC) on the growth of hyphae of *Aspergillus fumigatus* was studied by using this system. Although neither 2 μg/ml of AMPH nor 250 μg/ml of 5-FC alone showed any effect on the hyphal growth, their combination at these concentrations showed a distinct inhibitory activity. The biocell-tracer system is useful for antifungal activity testing in filamentous fungi.

Key Words——amphotericin B; *Aspergillus fumigatus*; biocell-tracer; 5-fluorocytosine; synergistic effect.

Developments in the fields of antibiotics, corticosteroids, and anticancer or immunosuppressive drugs, and the raising of standards of medical treatment since World War II have saved the lives of many patients with critical diseases. However, due to these developments, the number of compromised hosts has been increasing year by year. Opportunistic fungal infections are prone to occur in such patients, and in recent years the number of patients with deep mycoses has been increasing conspicuously.

To cope with this problem, it is necessary to measure the susceptibility of causative fungi to antifungal agents, and to this end several methods have been reported, including microdilution (NCCLS, 1992; Merz et al., 1984; Bezjak, 1985), agar diffusion (Kremer, 1979) and flow cytometry (Pore, 1990; Taguchi et al., 1992). These methods, however, are oriented mainly to yeasts and yeast-like fungi. For susceptibility testing of filamentous fungi to antifungal agents, conidium germination is used instead of hyphal growth, and for this purpose new methods for MIC testing in filamentous fungi are being sought.

Aspergillus fumigatus Fresenius is one of the three important causative agents of opportunistic fungal infections and its parasitic form in tissue is mycelial.

In 1990, Matsuoka et al. (1990) developed a new apparatus, the biocell-tracer, which automatically traces hyphal tips and determines their growth rate.

Several antifungal agents, including amphotericin B (AMPH), 5-fluorocytosine (5-FC), miconazole, fluconazole, and itraconazole, are used clinically. Although these drugs show considerable effects on causative fungi, it is relatively difficult to overcome systemic mycoses by single usage of these drugs. AMPH is considerably effective on systemic mycoses, but its toxicity cannot be disregarded. Therefore, combined administration of two or three antifungal agents with different action mechanisms is necessary for the treatment of serious mycoses.

In this work, we used the biocell-tracer system to examine the synergistic effect of AMPH and 5-FC on the hyphal growth of *A. fumigatus*.

Materials and Methods

Fungus *Aspergillus fumigatus* IFM 4942, isolated from a patient with invasive pulmonary aspergillosis, was used in this experiment. It was transferred to a fresh potato-dextrose agar (PDA: Difco, Detroit, U.S.A.) slant and incubated for 72 h at 25°C.

Medium For the testing of antifungal activity, 'synthetic amino acid medium, fungal' (SAAMF: Nihon Seibutsu Zairyo Co., Ltd., Japan) was used.

Antifungal agents AMPH in the form of Fungizone® (Bristol-Myers Squibb Co., Ltd., Japan) was dissolved in distilled water to a concentration of 1 mg/ml before use. Although Fungizone® contains sodium deoxycholate, no difference was found between sodium deoxycholate treated and untreated hyphae. 5-FC (Sigma Chemical Co., Ltd., St. Louis, Mo., U.S.A.) was dissolved in distilled water at a concentration of 10 mg/ml. Each antifungal suspension was further diluted with SAAMF and a proper concentration was prepared for inoculation.

MIC determination A previously described broth dilution method was used for the MIC assay with conidia of IFM 4942. A checkerboard titration was performed in

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which two-fold serial dilutions of AMPH (25 to 0.4 μg/ml) and 5-FC (800 to 0.8 μg/ml) were added to SAAMF in microdilution plates. Conidia (approximately 1×10^6 cells/ml final concentration) were added and the plates incubated for 48 h at 30°C. Amphotericin B powder was diluted in dimethylsulfoxide (less than 1% final concentration). End points were determined visually.

Preparation for monitoring by the biocell-tracer system Plastic tissue culture dishes (35 × 10 mm, Nunc, Denmark) were used as culture vessels. A sterile saline solution containing 0.05% (v/v) Tween 20 was gently poured onto a 3-day culture slant, and conidia of *A. fumigatus* were harvested by centrifugation. The conidia were washed 3 times with sterile distilled water by centrifugation and adjusted to 1×10^6 conidia/ml by use of a Thoma's hemocytometer. One μl (10^3 conidia) of the suspension was dropped into the center of a culture vessel bottom coated with 0.01% (W/V) poly-L-lysine (Sigma Chemical Co., Ltd., St. Louis, Mo., U.S.A.). The vessel was kept for 30 min at room temperature, then gently washed with 2 ml of sterile distilled water. By this procedure, conidia not adhering to the poly-L-lysine were washed out. After addition of 1 ml of SAAMF into the culture vessel, it was incubated for 24 h at 30°C. The colony obtained was 3 to 5 mm in diam. The culture vessel was set on the microscope stage and the medium was replaced with 1 ml of fresh SAAMF and allowed to stand for 20 min at 30°C. Then, an appropriate hypha that was growing horizontally on the vessel bottom was selected and monitored by the biocell-tracer system (Hidan Co., Ltd., Chiba, Japan). Its growth rate was measured for about 10 min.

Biocell-tracer system As shown in Fig. 1, the system consists of a culture vessel, a microscope (Olympus; IMT-2) and an image analyzer (Flovel) with a computer program system that traces a hyphal tip automatically. The chamber can be regulated at a temperature from 10 to 50°C. The apparatus can trace growing hyphal tips at speeds in the range of 0.5 to 20 μm/min. A hyphal tip was displayed on the TV monitor and data were recorded on a video-tape recorder (NEC) and analyzed in terms of several parameters.

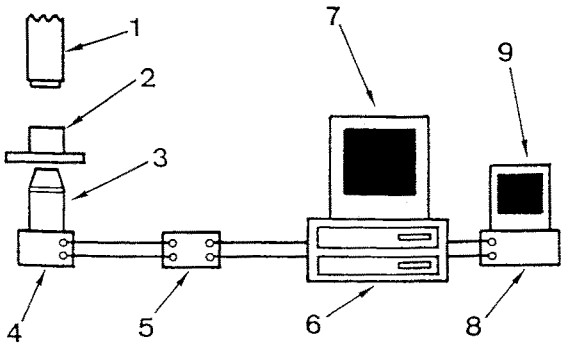

Fig. 1. Diagram of biocell-tracer system.
1. Light source; 2. Culture vessel; 3. Microscope; 4. CCD camera; 5. Image digitizer;6. Microcomputer; 7. Computer display; 8. Video tape recorder; 9. TV monitor.

Results

The MIC values of AMPH and 5-FC against *A. fumigatus* IFM 4942 determined by the dilution method were 12.5 μg/ml and 400 μg/ml, respectively. When either 2 μg/ml of AMPH or 250 μg/ml of 5-FC was used alone, no growth inhibitory activity was observed. However, when both drugs (AMPH; 2 μg/ml, 5-FC; 250 μg/ml) were added into the medium at the same time, a synergistic interaction was observed by the checkerboard titration method. On the basis of this result, we tried to examine the synergistic effect of the two drugs on a single hypha of *A. fumigatus* by using the biocell-tracer system. Experiments were repeated 9, 10 or 11 times in each assay system.

X and Y axes in Figs. 2 and 3 show times of measurement (min) and the growth rate (μm/min) of a single hypha, respectively. When the growth rate dropped to 0, we judged that the drug completely inhibited the hyphal growth. Fig. 2A shows an untreated control. The average growth rate of the hypha was constant at approximately 2.0 μm/min at 30°C. Figs. 2B and 2C show the growth rate of a hypha treated with 5-FC (250 μg/ml) and AMPH (2 μg/ml), respectively. Neither drug affected the growth rate. However, when both drugs were added simultaneously into the medium, the growth rate declined and finally dropped to 0 μm/min, as shown in

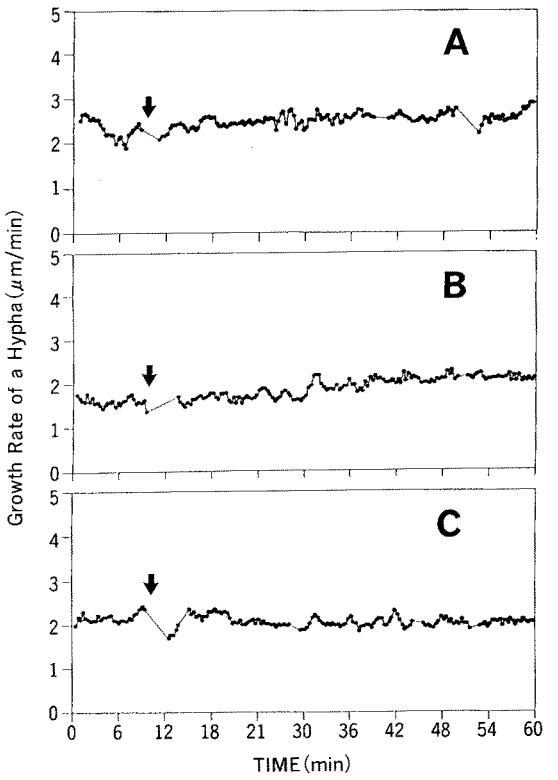

Fig. 2. Time course of the growth rates of individual hyphae of *A. fumigatus* IFM 4942.
Antifungal agents were added at the time indicated by the arrows. A: control (no addition). B: 5-FC (250 μg/ml, final concentration). C: AMPH (2 μg/ml, final concentration).

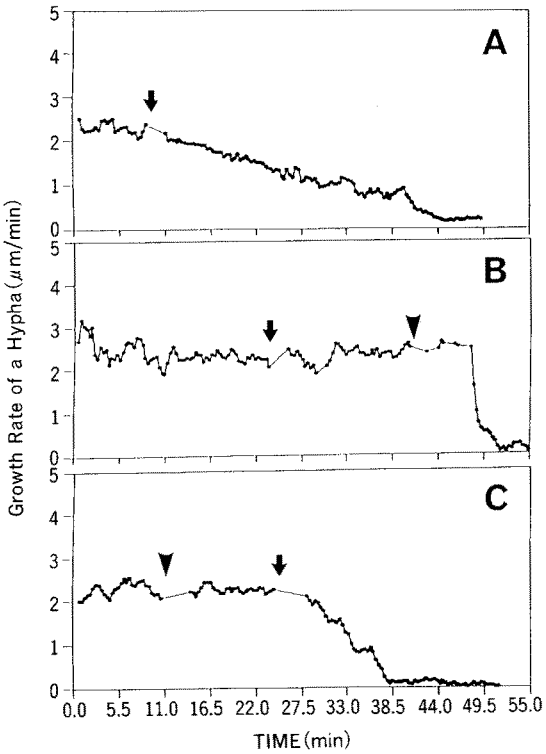

Fig. 3. Synergistic effect of 5-FC and AMPH on the growth rate of a single hypha of *A. fumigatus* IFM 4942.
A: 5-FC (250 μg/ml, final concentration) and AMPH (2 μg/ml, final concentration) were added simultaneously. B: 5-FC was added at the time indicated by the arrow, and AMPH was added at the time indicated by the arrowhead. C: AMPH was added at the time indicated by the arrowhead, and 5-FC was added at the time indicated by the arrow.

Fig. 3A. This phenomenon was observed for 7 out of 9 hyphae as shown in Table 1.

The synergistic effect of the two drugs was significantly influenced by the timing of their addition to the medium. When 250 μg/ml of 5-FC was added first into the medium and 2 μg/ml of AMPH was added 10 min later, the hyphal growth dropped immediately as shown in Fig. 3B. However, when AMPH was added first and 5-FC was added 10 min later, the hyphal growth dropped gradually as shown in Fig. 3C. Table 1 shows the synergistic effects on hyphal growth when the second drug was added 10, 20 and 30 min after the first drug. These results indicate that the synergistic effect of the two drugs was more pronounced when AMPH was used after 5-FC.

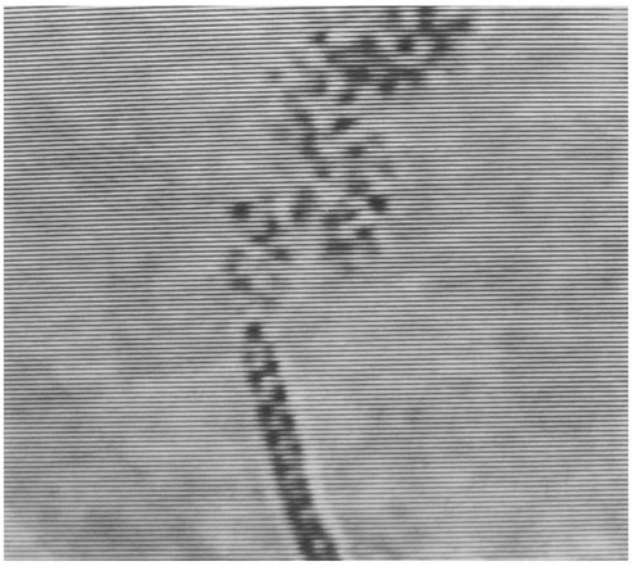

Fig. 4. A hypha treated with 5-FC and AMPH shows lysis.

When AMPH (2 μg/ml) and 5-FC (250 μg/ml) were used independently, no morphological changes of the fungal hyphae were observed. On the other hand, a combination of the two agents caused lysis of hyphae (Fig. 4).

Discussion

AMPH has a potent antifungal chemotherapeutic activity, even though it exhibits a high toxicity leading to such adverse effects as renal damage and hypopotassemia (Heidermann, 1983). In contrast, 5-FC has a low toxicity but gives rise to a resistance problem of the causative agent by continuous administration (Block et al., 1973). To achieve a satisfactory therapeutic effect against various fungal infections, a combination therapy of AMPH and 5-FC has been recommended by several researchers. However, no detailed strategy for the combination

Table 1. Time-dependent synergistic effect of AMPH and 5-FC on the growth of single hyphae of *Aspergillus fumigatus*.

	Drug acting time (min)			
	0	10	20	30
	Inhibition ratio (%)			
5-FC + AMPH (simultaneous addition)	78(7/9)[3]	—	—	—
5-FC (0 time) + AMPH (separate addition)[1]	—	90(10/11)	89(8/9)	89[4](8/9)
AMPH (0 time) + 5-FC (separate addition)[2]	—	89(8/9)	67(6/9)	44[4](4/9)

Drug concentration: Final concentrations of AMPH and 5-FC were 2 μg/ml and 250 μg/ml, respectively. 1) AMPH was added at indicated time. 2) 5-FC was added at indicated time. 3) Number of inhibited single hypha/Number of tested single hypha × 100 (%). 4) Statistically different ($p < 0.05$) by Student's *t*-test.

therapy has been reported (Corrado et al., 1982; Beggs and Sarosi, 1981). This experiment provided a new clue for developing a combination therapy of AMPH and 5-FC.

The biocell-tracer system is a suitable apparatus for studying the combination therapy of antifungal agents against filamentous fungus infections. As shown in this experiment, the synergistic effect of AMPH and 5-FC on *A. fumigatus* could clearly be monitored by use of this apparatus. However, several important points remain to be solved. One of them is how to stick hyphae on a culture vessel bottom without affecting hyphal growth. According to our preliminary experiment, poly-L-lysine was, as Crombie et al. (1990) had reported, a suitable substance for this purpose.

Murase et al. (1984) reported that a combination of 5-FC and AMPH led to an increase of 5-FC concentration in fungal cells compared to the single administration of 5-FC. On the other hand, Beggs et al. (1982) reported that incorporation of 5-FC into fungal cells was inhibited in the presence of AMPH. According to Beggs et al. (1981), 5-FC must be given before AMPH to obtain a greater synergistic effect. AMPH may have side-effects of inhibition of permease and deaminase in the fungal cell membrane, thereby allowing the increase of 5-FC concentration in fungal cells, as was reported by Beggs et al. (1982).

Our results suggest that it is possible to achieve a greater stability of synergistic effect by administering 5-FC first and AMPH there after (Table 1).

In this experiment, we succeeded in showing the synergistic inhibitory effect of AMPH and 5-FC on single hyphae of *A. fumigatus*.

MIC value usually expresses inhibition of fungal growth as a result of inhibition of conidium germination and subsequent hyphal elongation. On the other hand, the results obtained in this experiment indicate antifungal activity on the growth of single hyphae. Interestingly, the susceptibility of hyphae to these two drugs differs slightly from one hypha to another. This difference may be due to the culture age of each hypha.

Exposure to the drugs gave rise to interesting morphological changes in the hyphae, which the biocell-tracer system readily revealed. Exposure to 5-FC and AMPH gave rise to rapid lysis at the hyphal tip (Fig. 4). Therefore, the biocell-tracer system may also provide valuable information on the action mechanisms of antifungal agents.

In conclusion, the biocell-tracer system is a useful apparatus to study antifungal activities in filamentous fungi.

Literature cited

Beggs, W. H., Andrews F. A. and Sarosi, G. A. 1981. Combined action of amphotericin B and 5-fluorocytosine on pathogenic yeasts susceptible to either drug alone. Chemotherapy **27**: 247–251.

Beggs, W. H. and Sarosi, G. A. 1982. Further evidence for sequential action of amphotericin B and 5-fluorocytosine against *Candida albicans*. Chemotherapy **28**: 341–344.

Bezjak, V. 1985. Standardization of a hyphal inoculum of Aspergilli for amphotericin B susceptibility testing. J. Clin. Microbiol. **21**: 509–512.

Block, E. R., Jennings, A. E. and Bennett, J. E. 1973. 5-Fluorocytosine resistance in *Cryptococcus neoformans*. Antimicrob. Agents Chemother. **3**: 649–656.

Corrado, M. L., Kramer, M., Cummings, M. and Eng, R. H. 1982. Susceptibility of dematiaceous fungi to amphotericin B, miconazole, ketoconazole, flucytosine and rifampin alone and in combination. Sabouraudia **20**: 109–113.

Crombie, T., Gow, N. A. R. and Gooday, G. W. 1990. Influence of applied electrical fields on yeast and hyphal growth of *Candida albicans*. J. Gen. Microbiol. **136**: 311–317.

Heidermann, H. T., Gerkens, J. F., Spickard, W. A., Jackson, E. K. and Branch, R. A. 1983. Amphotericin B nephrotoxity in humans decreased by salt repletion. Am. J. Med. **75**: 476–481.

Koenig, H. and Kremer, M. 1979. A propos des discordances observees dans les resultats des C.M.I. faites sur spores ou filaments d'*Aspergillus*. Bull. Soc. Fr. Mycol. Med. **8**: 237–242.

Matsuoka, H., Ii, Y., Takekawa, Y. and Teraoka, T. 1990. Evaluation of antifungal volatile compounds on the basis of the elongation rate of a single hypha. Appl. Environ. Microbiol. **56**: 3779–3784.

Merz, W. G., Fay, D., Thumar, B. and Dixon, D. 1984. Susceptibility testing of filamentous fungi to amphotericin B by a rapid radiometric method. J. Clin. Microbiol. **19**: 54–56.

Murase, S., Yoshiyama, Y., Tomonaga, F. and Kume, H. 1984. Fundamental studies on combined effect by flucytosine and amphotericin B. Jpn. J. Med. Mycol. **25**: 125–138. (In Japanese.)

National Committee for Clinical Laboratory Standard(NCCLS). 1992. Reference method for broth dilution antifungal susceptibility testing of yeasts; Proposed standard. NCCLS document M27-P, Vol. **12** (25).

Pore, R. S. 1990. Antibiotic susceptibility testing of *Candida albicans* by flow cytometry. Current Microbiol. **20**: 323–328.

Taguchi, H., Tanaka, R., Miyaji, M., Nishimura, K., Kanno, H. and Ishiyama, H. 1992. Studies on the effect of combination of amphotericin B and flucytosine on *Candida albicans* by flow cytometry. J. Jpn. Assoc. Infect. Dis. **66**: 516–521. (In Japanese.)