In Vitro Susceptibilities of Human and Wild-Type Isolates of Basidiobolus and Conidiobolus Species

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The in vitro activities of amphotericin B, miconazole, ketoconazole, 5-fluorocytosine, and potassium iodide (KI) have been studied on human and wild-type isolates of Basidiobolus and Conidiobolus species. Of the antifungal agents tested, the imidazole derivatives, especially ketoconazole, were the most active against the agents of entomophthoromycosis. Transmission electron microscopy showed severe morphological alteration of Basidiobolus sp. exposed to 0.78 μg of ketoconazole per ml. The MIC and minimal fungicidal concentration of ketoconazole was often lowered in the presence of 10% fetal calf serum or antibiotic medium no. 3. Half of the Basidiobolus isolates and all Conidiobolus isolates were inhibited by amphotericin B at 0.39 μg/ml. None of the strains tested were inhibited or killed at maximum concentrations of 5-fluorocytosine and KI. The in vitro resistance of these fungi to KI at high concentrations suggests that the reported favorable treatment with KI may not be due to its direct effect on these fungi but rather to other, undefined factors in combination with KI. These data suggest that ketoconazole may be of use in the treatment of entomophthoromycosis, particularly in cases which are not responsive to KI.

Basidiobolus and Conidiobolus species (Entomophthorales) cause a chronic, inflammatory, granulomatous disease collectively called entomophthoromycosis (10, 12, 13, 24). The disease is generally restricted to the subcutaneous tissue or the nasal submucosa, but involvement of deeper structures such as the lungs, liver, muscles, and gastrointestinal tract in entomophthoromycosis basidiobolae and the paranasal sinuses in entomophthoromycosis conidiobolae have occurred (18). Although seldom life threatening, these diseases can cause severe disfigurement. The disease is prevalent in tropical and subtropical regions of the world, primarily in certain parts of Africa and southeast Asia (4), and was recently reported in Latin America (2, 15). Rare cases of entomophthoromycosis have been reported in the United States (8, 9, 23). The treatment of choice is potassium iodide, which is not specific; failures have been reported (14, 18). This study evaluates the in vitro activity of amphotericin B, miconazole, ketoconazole, 5-fluorocytosine, and potassium iodide on Basidiobolus and Conidiobolus isolates. (This paper was presented in part at the 83rd Annual Meeting of the American Society for Microbiology [B. G. Yanco, J. I. Okafor, and D. T. Wagner-Merner, Annu. Meet. Am. Soc. Microbiol. 1983, F19, p. 385].)

MATERIALS AND METHODS

Fungal isolates. Human isolates of Basidiobolus species (B. meristosporus ATCC 36600, B. ranarum ATCC 14052 and ATCC 24670, and B. haptosporus ATCC 34122), three human isolates of Conidiobolus species (C. coronatus ATCC 42063 and ATCC 32867 and C. incongruus ATCC 24293), and wild-type isolates of Basidiobolus species (B. ranarum ATCC 32277, B. magnus ATCC 15379, and B. microsorum ATCC 14708) were obtained from the American Type Culture Collection, Rockville, Md. Wild-type isolates of Basidiobolus species (L21, L24, L25, L27) were gathered from lizard guts in Nigeria, and GA5 and T1 were obtained from a lizard gut and a toad, respectively, in Florida. Candida albicans with known susceptibility to various antifungal agents was used as the control organism.

Antifungal drugs. Amphotericin B was purchased from E. R. Squibb & Sons, Princeton, N.J. (NDCC03-0437-30). 5-Fluorocytosine was kindly provided by Hoffmann-LaRoche, Inc., Nutley, N.J. (lot no. 134119). Miconazole (lot no. B02/1) and ketoconazole (lot no. C6401) were generously provided by Janssen Pharmaceutica, Inc., New Brunswick, N.J. Potassium iodide (KI) was purchased from Mallinckrodt, Inc., St. Louis, Mo. The solutions were prepared according to the instructions of the respective manufacturers.

Media. Unbuffered yeast-nitrogen base (YNB), antibiotic medium no. 3 (U.S. Food and Drug Administration), Sabouraud dextrose broth (SDB) and agar (SDA) (Difco Laboratories, Detroit, Mich.), and Eagle minimum essential medium (EMEM) supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) were utilized for the study.

Preparation of inocula. All isolates were grown on SDA at 30°C for 48 to 72 h to obtain young and actively growing cultures consisting of only mycelia and conidia without zygosporangia. Fungal scrapings were suspended in sterile saline and vortexed until an almost-uniform suspension of small fragments of fungi was observed. From this, an adjusted fungal suspension of 10⁴ to 10⁵ cells per ml counted in a hemacytometer chamber was used as the source of inoculum.

Broth dilution method. The macro-broth dilution method of Shadomy and Espinel-Ingroff (19) was used in this study. To each dilution of antifungal agents ranging from 0.98 to 200.0 μg/ml, 0.1 ml of a fungal suspension was added. For KI, a maximum concentration of 30,000 μg/ml was used. These and the controls were incubated at 30°C for 48 h. The MIC was defined as a tube in which no visible growth, as compared with the control, could be seen. The minimal fungicidal concentration (MFC) was defined as a tube in which no growth occurred 48 h after being subcultured on a
plate of SDA. Approximately 0.075 ml was sampled and subcultured from tubes with no visible growth. All experiments were performed in triplicate. All drugs were tested against a single isolate at one time.

**Electron microscopy.** *Basidiobolus* sp. (GA5) in 0.78 μg of ketoconazole per ml and an untreated control were incubated at 30°C for 48 h and then fixed in 3% distilled glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0. These specimens were stored at ca. 4°C for 48 h, after which they were rinsed in the same buffer and post-fixed in OsO₄ for 1 h. Subsequently, the specimens were rinsed in buffer and dehydrated in a graded ethanol series, followed by 100% acetone (6). Spurr (21) low-viscosity embedding medium was used, with hypafex flatly embedded. Areas selected for sectioning were cut out, mounted on microtome stubs, and thin-sectioned with a Sorvall MT-2 ultramicrotome (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). The sections were stained for 15 min in 0.5% aqueous uranyl acetate and in lead citrate for 5 min (17) before examination with a JEOL 100-C electron microscope (Japan Electron Optics, Peabody, Mass.).

**RESULTS**

**Susceptibility to antifungal drugs.** The MICs and MFCs of various antifungal drugs for 13 *Basidiobolus* and 3 *Conidiobolus* isolates are shown in Table 1. The MICs of amphotericin B for 50 and 90% of the isolates (MIC₃₀ and MIC₉₀) were 0.39 and 0.78 μg/ml, respectively. At 0.39 μg of amphotericin B per ml, only 31% of *Basidiobolus* isolates were killed. The MIC₃₀ and MIC₉₀ of miconazole were 0.39 and 3.12 μg/ml, respectively. Of the total, 11 isolates (85%) were killed at 3.12 μg/ml. The MIC₉₀ of ketoconazole was ≤0.098 μg/ml. All isolates were inhibited and killed by ketoconazole at a concentration of 1.56 μg/ml. None of the isolates were inhibited or killed at the highest concentrations used for 5-fluorocytosine and KI.

Amphotericin B inhibited *C. coronatus* ATCC 32867 and *C. incongruus* ATCC 24293 at 0.098 μg/ml, whereas *C. coronatus* ATCC 42063 was inhibited at 0.39 μg/ml. The MFC of amphotericin B for ATCC 32867 and ATCC 24293 was 0.098 μg/ml, whereas ATCC 42063 was killed at 0.78 μg/ml. The latter isolate required a higher MIC and MFC of miconazole (3.1 and 12.5 μg/ml, respectively), whereas ATCC 32867 and ATCC 24293 required an MIC and MFC of miconazole of 0.098 to 0.195 μg/ml and 0.195 μg/ml, respectively. The MICs and MFCs of ketoconazole for ATCC 42063, ATCC 32867, and ATCC 24293 were 1.56, 0.195 and 0.78, and 0.195 and 0.39 μg/ml, respectively. Both *Conidiobolus* species were not inhibited or killed by 5-fluorocytosine and KI at maximum concentrations tested.

**Effect of media on susceptibility to ketoconazole.** The effects of different media on the MIC and MFC of ketoconazole to some of the *Basidiobolus* isolates are shown on Table 2. The addition of 10% fetal calf serum to SDA and the use of EMEM with 10% fetal calf serum or antibiotic medium no. 3 lowered the MICs and MFCs of ketoconazole by eight times for ATCC 36600, ATCC 14052, and ATCC 32277. However, an opposite effect was observed with ATCC 14708 and ATCC 15379. The MICs and MFCs obtained when YNB was used were comparable to those obtained when SDB without fetal calf serum was used. The wild-type isolates were tested only with YNB and antibiotic medium no. 3. Again, lower MICs and MFCs of ketoconazole were observed when antibiotic medium no. 3 was used for testing these wild-type isolates.

**Electron microscopy.** Electron microscopy of a normal cell of *Basidiobolus* GA5 is shown in Fig. 1A, and a cell exposed to 0.78 μg of ketoconazole per ml is shown in Fig. 1B. These latter cells are in a necrotic state in which no cell organelles are definable.

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<th>TABLE 1. Effects of various antifungal drugs on <em>Basidiobolus</em> and <em>Conidiobolus</em> isolates</th>
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<td><strong>Drug</strong></td>
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<td>Amphotericin B</td>
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<th>TABLE 2. Effect of type of medium on the MIC and MFC of ketoconazole for some isolates of <em>Basidiobolus</em> species</th>
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<td><strong>Isolate</strong></td>
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* a CS, 10% Fetal calf serum.  
b AM3, Antibiotic medium no. 3.  
c —, Not done.
**DISCUSSION**

Our study showed that imidazole derivatives are active against the etiological agents of entomophthoromycosis. The highest MIC and MFC of miconazole for the majority of isolates was 3.12 μg/ml, and the highest MIC and MFC of ketoconazole for all isolates was 1.56 μg/ml. These are within achievable therapeutic serum levels, which are 7 to 8 μg/ml after a 600- to 1,000-mg intravenous dose of miconazole (22) and 2 to 4 μg/ml after a 200-mg oral dose of ketoconazole (3). Drug levels in serum of up to 50 μg/ml, if needed, may be achieved by higher doses of ketoconazole (3). The in vitro effect of ketoconazole was corroborated by electron microscopic evidence of morphological obliteration of cellular structures in a Basidiobolus strain exposed to this drug. Our results showing variations of MICs and MFCs of ketoconazole in different media were comparable to those obtained in a previous study (1) in that a lower MIC was observed when 10% calf serum was added to SDA or when EMEM with 10% calf serum was used. In our study, however, there appeared to be no significant difference among the MICs and MFCs of ketoconazole obtained with EMEM, SDA with 10% calf serum, and antibiotic medium no. 3.

Amphotericin B was active against only 50% of the Basidiobolus isolates we tested at 0.39 μg/ml, which is the concentration accepted as the limit point for susceptibilities of fungi to this drug (18). Likewise, one of our three Conidiobolus isolates (ATCC 42063) also showed resistance to amphotericin B. These results substantiate reports of clinical failure of amphotericin B in the treatment of entomophthoromycosis basidiobolae (18) and entomophthoromycosis conidiobolae (11, 14–16).

Maximum concentrations of 5-fluorocytosine and KI did not show any activity against the isolates tested. As with amphotericin B, 5-fluorocytosine has failed previously in the treatment of entomophthoromycosis (11). The disparity between in vitro observations and the known in vivo activity of KI in entomophthoromycosis is of interest. In our in vitro results were comparable to those in earlier studies by Tio and deVries (25), who felt that KI activity in this disease is not due to a direct effect on the fungus but rather to nonspecific action. A similar phenomenon has been observed with Sporothrix schenckii, in which initial in vitro studies failed to demonstrate any direct activity of KI against this fungus (5, 7) despite its high clinical efficacy in the treatment of sporotrichosis. Early investigators (5) suggest that KI stimulates the healing process in sporotrichosis. Others (20) have felt that molecular iodine may enhance phagocyte activity. Additional studies suggest that free or molecular iodine formed from the body may be fungicidal, whereas KI may be fungistatic (26, 27). However, the in vitro iodide concentrations necessary to attain antifungal effects were much greater (≥4 times) (26) than achievable levels in serum (ca. 50 μg/ml), to explain a direct effect in vivo. Therefore, the exact mechanism of action of KI in sporotrichosis and entomophthoromycosis still remains to be elucidated.

This study suggests that the imidazole derivatives, particularly ketoconazole (an orally administered drug), may be effective in the treatment of entomophthoromycosis and may be considered a potential alternative therapy, especially in patients who failed to respond to KI therapy. However, clinical studies are needed to confirm our in vitro observations.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**

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