ANTIMYCOTIC SUSCEPTIBILITY OF THE CONIDIAL AND HYPHAL PHASES OF ASPERGILLUS FUMIGATUS BY CONVENTIONAL AND NEW METHODOLOGY

by

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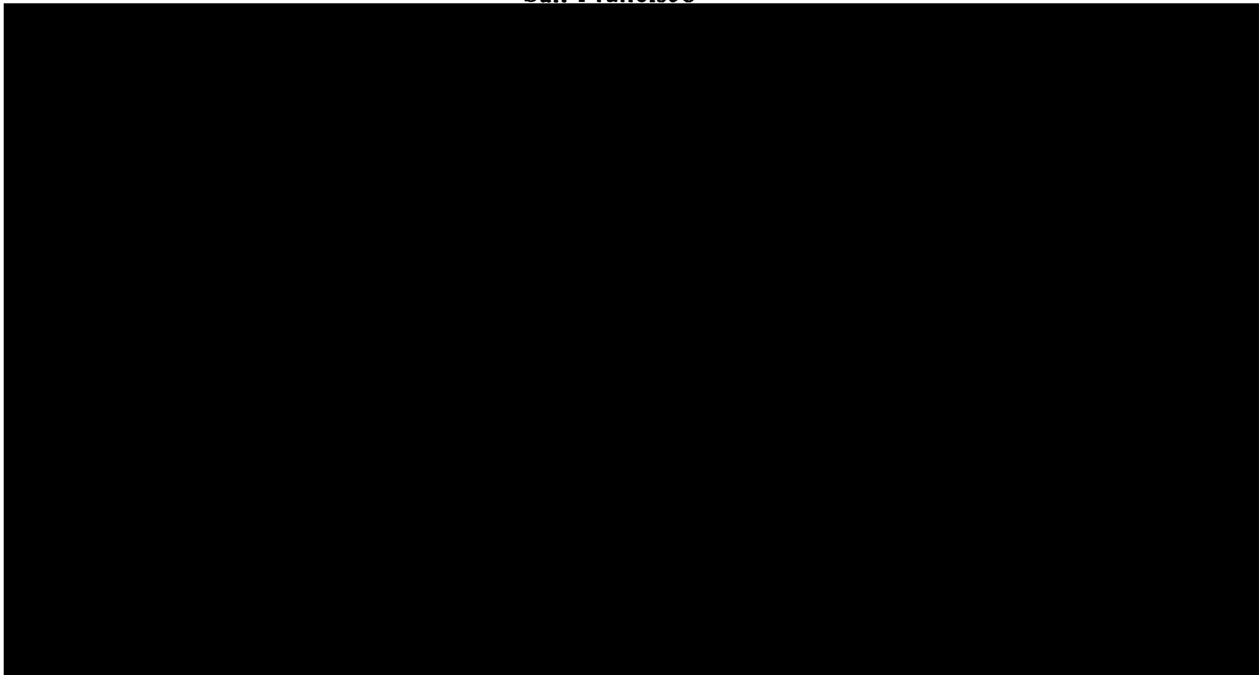

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INTRODUCTION

Aspergillus infections in man can occur in several sites in the body and may cause either serious systemic or superficial infection (1 - 3). Aspergillosis is an increasing cause of mortality in patients with underlying diseases such as cancer or diabetes. It also presents life-threatening problems after severe burns and in autoimmune diseases. Iatrogenically and nosocomially produced aspergillosis often occurs during immunosuppresive drug therapy (2, 4).

The three most commonly encountered forms of aspergillosis following infection with *Aspergillus fumigatus* are described:

1. Allergic or non-invasive type includes atopic bronchopulmonary disease with pulmonary infiltrates and eosinophilia. This type may take the form of an asthmatic allergy to inhaled spores. It can become chronic and progressively severe, thereby causing bronchitis and bronchial occlusion (2). Pathologic changes that result are due in part to an immediate and delayed type hypersensitivity reaction to the fungal spores in the bronchi (2).
2. Invasive type aspergillosis involves necrosis of the bronchi and invasion of small blood vessels and surrounding tissue (4). Dissemination of mycelia from the lung to other organs has been reported to occur in leukemic patients (4).
3. Aspergilloma, the fungal colonization of a cavity in the lung, is the third type of disease caused by *A. fumigatus*. Cavities formed from an ectatic bronchus contain a

tangled mass of mycelia (2). Commonly these fungus balls occur in old tuberculosis cavities (2). If an air space is present in the aspergilloma, the fungus may produce fructification structures. But the more typical histological finding is the characteristic dichotomous branching, radiating, septate hyphae oriented in the same direction (1, 2).

Although inhalation of spores may initiate infection in the respiratory tract (5), hyphae are the invasive morphological form in asperillosis and are, therefore, of prime clinical importance and of special interest in drug susceptibility studies.

The course of infection has been studied in mice by Ford and Friedman (6) and Sidransky and Epstein (7, 8). They used spores that were either inhaled by mice or injected intraperitoneally; spores subsequently ingested by alveolar or peritoneal macrophages were examined. In normal mice, the spores did not germinate in macrophages but were not necessarily killed. However, when cortisone was given to mice prior to challenging them, the spores were observed to germinate intracellularly. When the animals were infected with germinated spores, a higher incidence of lethal infections occurred than when non-germinated spores were used. The animals that died from lethal aspergillosis presented inflammatory infiltrations and hyphal invasion in visceral organs (7).

The above considerations on the role of morphologic growth phases in infectious mycotic processes present problems unique to determinations on fungal drug susceptibility. For, whereas infections are initiated, for example, with _Aspergillus_ spores (conidia),

pathologic sequelae result from mycelial invasion. The objective of this study was to investigate antimicrobic susceptibility of fungi which initiate infections by the spore form but which produce a subsequent disease process by the mycelial form. Such mycoses, superficial or deep, include those caused by opportunistic fungi such as Aspergillus, Mucor and the dermatophytes. They represent the most frequently encountered infections of fungal origin. For reasons outlined below, attention was focused on the synthetic antimicrobic, miconazole.

Treatment of aspergillosis, until recently, has been limited to amphotericin B, 5-fluorocytosine and/or surgical resection (4, 9). Results varying from clinical cure to failure, often depending upon underlying disease of the patients, have been described (10). Amphotericin B, although successfully used in many systemic and opportunistic infections, is a nephrotoxic antimicrobic (11). 5-Fluorocytocine is inhibitory only to a narrow spectrum of susceptible fungi, notably some of the pathogenic yeasts, but emergence of resistant populations of organisms limits its usefulness (12, 13). The problem of reliable antifungal therapy remains formidable.

In 1972, miconazole (MCZ), a non-toxic antifungal agent effective against a broad spectrum of fungi, was introduced by Van Cutsem and Thienpont (14). MCZ is a β-substituted phenethyl imidazole, with a molecular weight of 479.16 which when dissolved in Cremophor El (polyethoxylated castor oil) is soluble in water. It is very stable at room temperature and 4°C and readily diffuses in Sabouraud glucose agar (SAB). Resistant populations emerging in vitro have not been demonstrated (14, 15).

The mechanisms of its inhibitory actions appear to be dependent

somewhat on dose levels (16). Studies by De Nollin and Van Belle on yeast cells of *Saccharomyces cereviseae* and *Candida albicans* revealed several effects. At fungicidal dose levels, the following were noted: 1) a decrease in cytochrome c oxidase enzyme on mitochondrial cristae, 2) inhibition of cytochrome c peroxidase and catalase enzymes, and 3) a decrease in alkaline phosphatase activity. It is suggested that MCZ interferes with the synthesis of these enzymes, rather than having a direct effect on the enzyme activity (16).

Van Den Bossche (17, 18) studied the influence of MCZ on the cellular permeability barrier of *Candida albicans*. MCZ interfered with ergosterol synthesis, an important component of cytoplasmic nuclear, mitochondrial and vacuolar membranes of yeast and other fungi. Leakage of inorganic and organic cell components occurred across the defective cellular membrane. It was postulated that after exposure of the yeast cells to a minimal fungicidal dose of MCZ, the inhibition of ergosterol synthesis and the formation of peroxides in the cell may have accounted for the lethal effects of the drug (16, 17, 18, 19).

The use of MCZ in the therapy of systemic mycoses was recently reviewed (20, 21, 22, 23). Although many strains of *A. fumigatus* have been shown to be resistant in vitro, cures as well as failures in aspergillosis have been seen with intravenous treatment (24). Because MCZ appears to be a drug of promise in the treatment of systemic and superficial mycoses and it possesses favorable properties of stability, solubility and broad spectrum application in vitro, it was chosen for this study (23).

In vitro antibiotic susceptibility testing of bacteria is now well standardized (25). Although antimycotic studies on yeasts have

employed techniques similar to those used with bacteria (26, 27), a review of methods currently used to assess fungal susceptibility to drugs indicates clearly that standardized procedures have not yet been developed (28, 29, 30, 31). A great variety of techniques have resulted in widely differing drug susceptibility profiles. Susceptibility patterns of dimorphic fungal pathogens such as *Histoplasma capsulatum* and *Coccidoides immitis* have been investigated using a number of techniques, only some of which were concerned with the differential susceptibilities of the different growth phases (32, 33, 34, 35, 36). Less work has been done on the opportunistic filamentous fungi, especially *A. fumigatus*, which produces hyphal and occasionally spore forms in diseased tissue.

Drug suceptibility testing of filamentous fungi pose special problems over and above those important in antibiotic testing of bacteria, such as length of exposure to antimicrobic, temperature of incubation, concentration of drug and composition of culture medium. A critical factor in antimycotic studies is the nature of the fungal inoculum. Fungi frequently assume different morphological forms in vitro and in vivo. The particular form that is chosen for determining in vitro drug susceptibility must relate to the morphological form(s) that occur in diseased tissue. It is these forms, ultimately that must be controlled by antifungal drugs if a therapeutic advantage is to be realized. With respect to the fungal inoculum alone, significant differences in the minimum inhibitory concentration (MIC) for yeast and mycelial or spore and mycelial phases have been recognized (33, 37, 38).

Cheung and Medoff (39) and later Cheung and Kobayashi (33) found

differing susceptibility responses in the yeast and mycelial phases of H. capsulatum to combinations of cyclohexamide and chloramphenicol, and amphotericin B and actinomycin D, respectively. Yeast cells were ten times more resistant than mycelia to amphotericin B (AMB) as determined by the lack of incorporation of ^{14}C guanine into RNA in the presence of the drug (39).

Collins and Pappagianis (34) found the inhibitory levels of AMB for the spherule-endospore phase of C. immitis (Silveira strain) to be four- to eight-fold lower than the mycelial phase in an agar dilution test. Levine and Stevens (32) demonstrated that mycelial fragments of two strains of C. immitis were inhibited by doses of MCZ two- to three-fold higher than the doses inhibitory to the endospores. Brandsberg and French (37) observed that A. fumigatus mycelia gave a higher MIC value than conidia to AMB in a broth dilution test. However, Kitahara and Seth (30) found no difference between germinated or ungerminated conidia of three strains of A. fumigatus by a broth dilution test.

Berger and Merz (40) studied the response of three isolates of A. fumigatus to various incubation temperatures, 23°, 37° and 45° C, in the presence of cyclohexamide. The effect of temperature on susceptibility was observed at the lowest temperature, 23° C, which produced the most significant inhibition of growth. The higher temperatures, 37° and 45° C, showed much less inhibition. The reason for increased tolerance to cyclohexamide at higher temperatures was unexplained.

The effect of media components on the action of antifungal drugs is controversial. Wagner and Shadomy (41) reported antagonism between 5-fluorocytosine and purine and pyrimidines which significantly altered

the MIC values of 7 strains of aspergilli. Hoeprich and Huston (42) studied four culture media, SAB, brain heart infusion (BHI), modified yeast-nitrogen base (YNB) and SAAMF (synthetic amino acid medium, fungal) (43) and their effect on MCZ, clotrimazole (CLO), amphotericin B methyl ester (AMBME) and AMB in MIC determinations with Candida albicans, C. parapsilosis, Torulopsis glabrata and Cryptococcus neoformans. They found antagonism between both imidazoles, MCZ and CLO, and SAB and BHI. It was assumed that these results were due to the presence of purines, pyrimidines and nucleosides in these two undefined media. However, addition of these components to the most well defined medium, SAAMF, did not alter the MIC results obtained with SAAMF. The difference between results in the undefined complex media, SAB and BHI, and the defined synthetic media, SAAMF and YNB, is unexplained.

In contrast, Collins and Pappagianis (34) using a SAB agar dilution method, tested 4 strains of C. immitis, previously determined by Hoeprich and Huston (42) to vary widely in susceptibility to AMB. Collins and Pappagianis found the 4 strains to be similar in susceptibility over a 7 day incubation period. They also determined that the antifungal action of AMB was not inhibited by complex undefined medium.

It is evident in the above studies that diverse factors are responsible for the susceptibility results. This study was designed to determine the effects of a basic and critical factor, intrinsic in any in vitro antimycotic susceptibility study, that of the fungal inoculum. In order to understand the differential susceptibilities of fungal growth phases, MIC data on mycelia and spores are pertinent to the drug suceptibility issue.

MATERIALS AND METHODS

Media

The growth medium used for all drug susceptibility comparisons was Difco Sabouraud glucose (SAB) broth solidified with 1.5% of Bacto agar. The pH after autoclaving was 6.0 ± 0.2. Aliquots of 55 ml of SAB agar were added to Falcon 150 x 75 mm plastic petri dishes to obtain a depth of 4 mm for the angle diffusion (ADM) assays. Sixty-five ml of SAB agar was employed as the base layer for agar diffusion assays. Nine ml of SAB (0.75% agar), to which 1 ml of either spore or germling broth suspension was added, was used as the overlay for the agar diffusion assay (Figure 1). Broth dilution assays were performed in 1.8 ml SAB broth to which 0.2 ml of the appropriate MCZ concentration and 0.1 ml of either spore or germling broth suspension was added.

Czapek agar (44) and glucose yeast extract agar (2% glucose, 1% Difco yeast extract, 2% Difco agar) (GYE) was used to harvest the conidial phase of certain *A. fumigatus* strains. The conidia were germinated in SAB broth. All media were stored at 4° C until used. Prior to inoculating SAB with these conidia, the medium was supplemented with filter sterilized, aqueous 0.001% Triton X-100 (Beckman Instruments, Inc., Fullerton, CA.). This detergent aided in dispersing clusters of conidia and germlings (8 hour germinated conidia) (45) when diluting them for the test inoculum.

Cultures

A. fumigatus cultures were obtained primarily from patients with diagnosed aspergillosis. Identification of each strain was confirmed

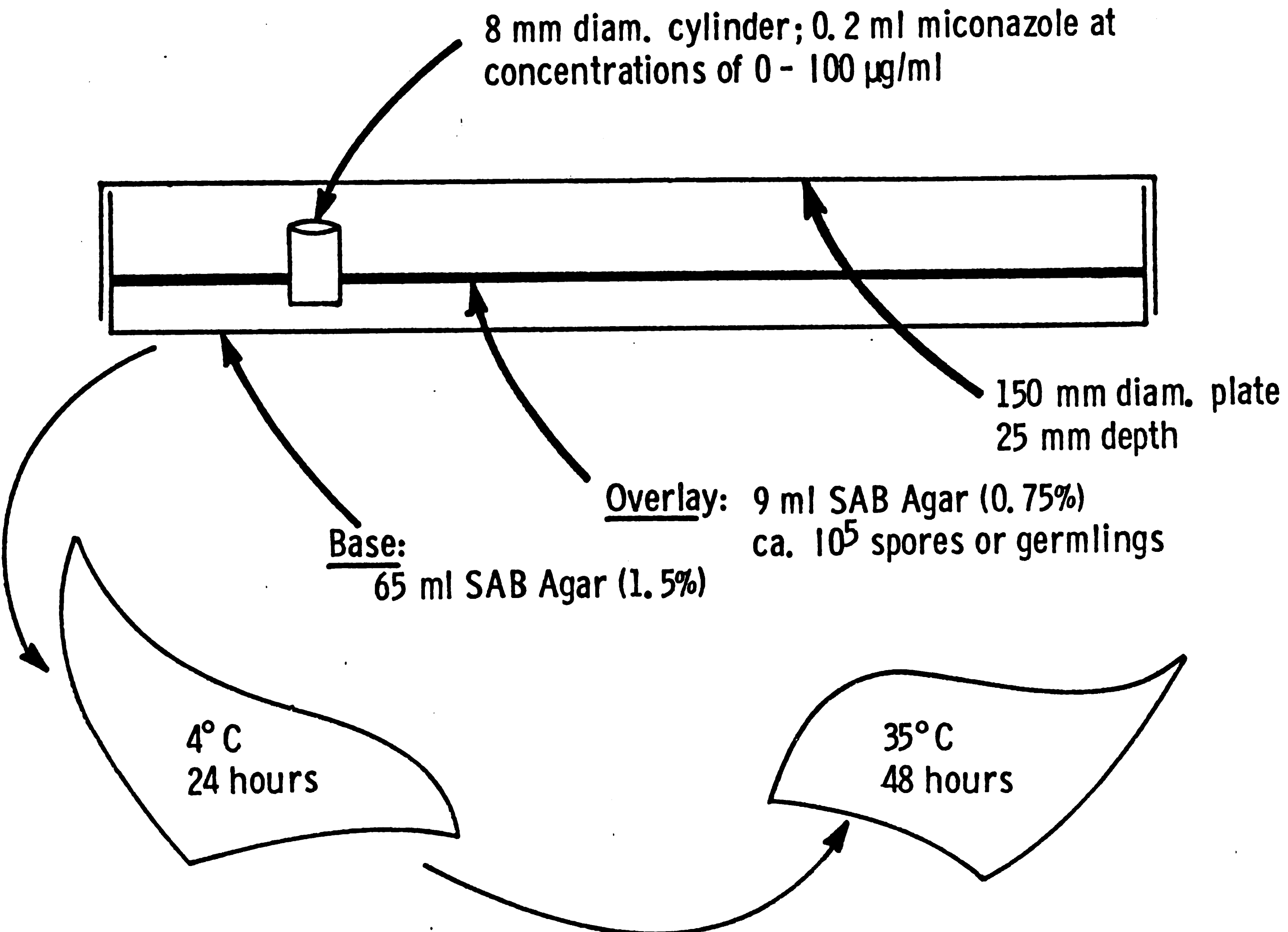

Figure 1. Agar diffusion method.

using characteristics described by Raper and Fennell (46). Cultures originated from lung, bronchus, sputum, ear and endometrium specimens (Table 1) and were kindly supplied by the mycology laboratories of San Francisco General Hospital, Microbial Diseases Laboratory of the California State Department of Health Services and the Naval Biosciences Laboratory of the University of California at Berkeley.

Table 1. Clinical Sources of *A. fumigatus* Cultures

Number of isolates	Source
11	Sputum
3	Bronchoscopy, bronchial wash
5	Lung biopsy, chest tube
1	Pleural fluid
1	Ear
1	Endometrium

Separate suspensions of conidia and germinated conidia from each strain were obtained as follows:

Conidia. Large (150 X 75 mm) petri dishes containing 25 ml of SAB, Czapek or GYE agar were inoculated with a conidial and hyphal suspension over the surface of the agar. The plates were incubated at 35° C for 5 to 7 days after which abundant spore production was observed. Sterile 5 mm diameter glass beads were gently rolled over the surface of the growth until they were coated with a layer of conidia and then transferred to 5 ml of sterile

saline. The viability of spores remained stable for 16 months at 4° C (47). The suspensions were employed as stocks and were also the source of germlings. Conidial suspensions for inhibition studies were first prepared by diluting the stock suspension in 9.5 ml SAB broth (to which 0.1 ml of sterile 0.1% Triton X-100 was added) to the point of vanishing turbidity, using a Wickerham card (26). This corresponded to a concentration of 10^5 to 10^6 conidia per ml. Viable numbers were determined by conventional surface plate counting technique on SAB and by hemocytometer counts. In later studies, inocula were standardized spectrophotometrically to 95% transmittance on a Spectronic 20 at 420 mμ which corresponded to 10^5 to 10^6 viable conidia per ml.

<u>Germlings</u>. Conidial suspensions, prepared as above, were germinated in broth at 35° C for 8 hours or at 25° C for 18 hours. Germination was checked microscopically for the typical appearance of germlings (Figure 2).

<u>Antimicrobic</u>

Miconazole (MCZ) (Janssen Pharmaceutica, Beerse, Belgium) (Figure 3) was supplied as a sterile, aqueous colloid, an intravenous formulation containing 10 mg/ml MCZ, 0.5 mg/ml sodium bisulfite, 1.62 mg/ml methyl paraben, 0.18 mg/ml propyl paraben, 0.115 ml Cremophor EL (polyethoxylated castor oil), sodium acetate and acetic acid for pH adjustment to 4 $\pm$ 0.5. MCZ was diluted in sterile distilled water to concentrations ranging from 1000 μg/ml to 1.6 μg/ml

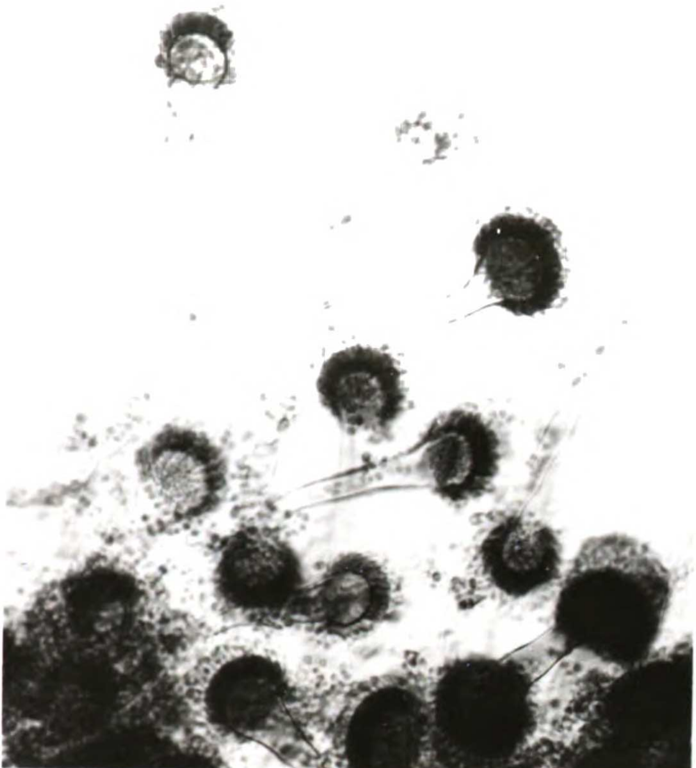

Figure 2A. Conidial heads of Aspergillus fumigatus (x 100).

Figure 2B. Germinated conidia of Aspergillus fumigatus in Sabouraud glucose broth at 8 hours, 35° C (x 400).

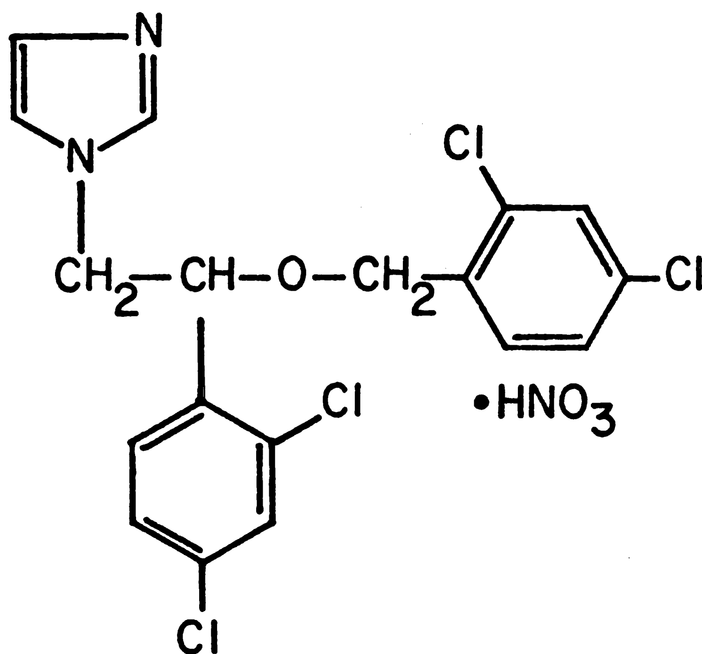

Figure 3. Structure of miconazole.

and stored at 4° C. Diluted MCZ is stable at 4° C for at least 2 months (48). Fresh dilutions were prepared every 2 months and potency was checked periodically with control cultures.

In order to assure that the drug solutions were active and that the test conditions were not excessively discordant on different days, a control culture, _A. fumigatus_ strain 3, was included in each assay.

The effect of inoculum size on the MIC value was determined in early broth dilution studies. _Aspergillus_ spores were diluted in SAB broth to final concentrations of 10^3, 10^4, 10^5, and 10^6 spores per ml. Each of the 4 spore dilutions were tested with MCZ concentrations of from 1.6 to 100 µg/ml for 48, 72 and 96 hours of incubation.

For the broth dilution method, dilutions of MCZ were made by adding 0.2 ml of drug solution to 1.8 ml of SAB broth to obtain final concentrations of from 0.8 µg/ml to 100 µg/ml. In studies using the agar diffusion method, 0.2 ml of MCZ, ranging in concentration from 1.6 µg/ml to 100 µg/ml, were added to each stainless steel cup (8 mm O.D. diameter) (Figure 1). Susceptibility studies using the angle diffusion method employed 0.2 ml of 100 µg/ml MCZ in the drug trough.

Instrument and Methodology for Angle Diffusion Studies

A device was designed for cutting two uniform elliptical troughs in agar, each 56 mm long by 4 mm wide, adjustable from 25° to a 50° angle from one another (Figure 4). The cutter was constructed of sharpened stainless steel narrow ellipses in an enclosed housing which was fitted with leads to a vacuum source. When the cutter was placed on the agar surface and then depressed into the agar, the housing was sealed and the agar ellipses could be removed by vacuum. The cutter

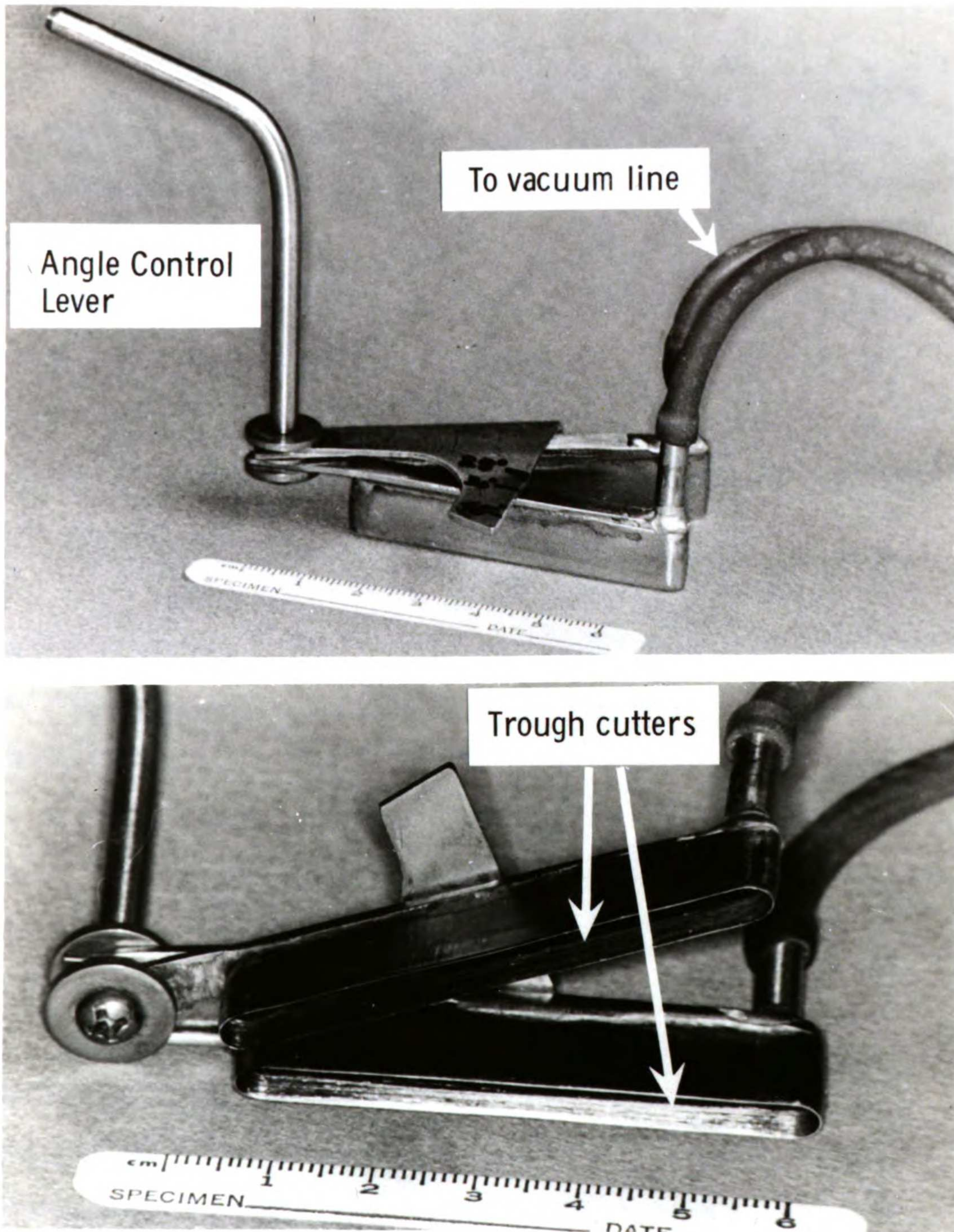

Figure 4. Trough cutting instrument employed in the angle diffusion method (ADM).

was alcohol-flamed before use. The pattern of the troughs in agar is shown in Figure 5.

Trough A acted as a reservoir for a suspension of fungal cells and trough B was a reservoir for the drug solution. The elongated elliptical shape of trough B allowed a linear diffusion front along and at right angles to its length. A pattern of diffusion studied with aqueous dyes in trough B showed that its advancing front intersected trough A in a manner that produced a gradient dye concentration at the second trough. The dye was most concentrated at the point where the two troughs were closest; it was least at the distal arms of the troughs.

In the ADM studies, MCZ solution was added to trough B and conidia to trough A. The drug was first allowed to prediffuse into the surrounding agar for 24 hours at 4° C. The plates were then incubated 48 hours at 35° C during which time drug diffusion continued but fungal growth was initiated now because of the elevated temperature. An even front of mycelia grew out of the inoculum trough and a measurable growth pattern was evident at 48 hours when inhibition measurements were recorded.

In all ADM assays, the angle between the troughs was set at 25°. The diffusing drug caused a concentration gradient at right angles to its trough as in conventional disc or cup tests (35). However, because the two troughs were not equidistant along the lengths of their arms, a steeply falling continuous drug gradient formed in the agar between troughs. As the distance between troughs increased, the drug concentration in the agar between troughs decreased. Thus,

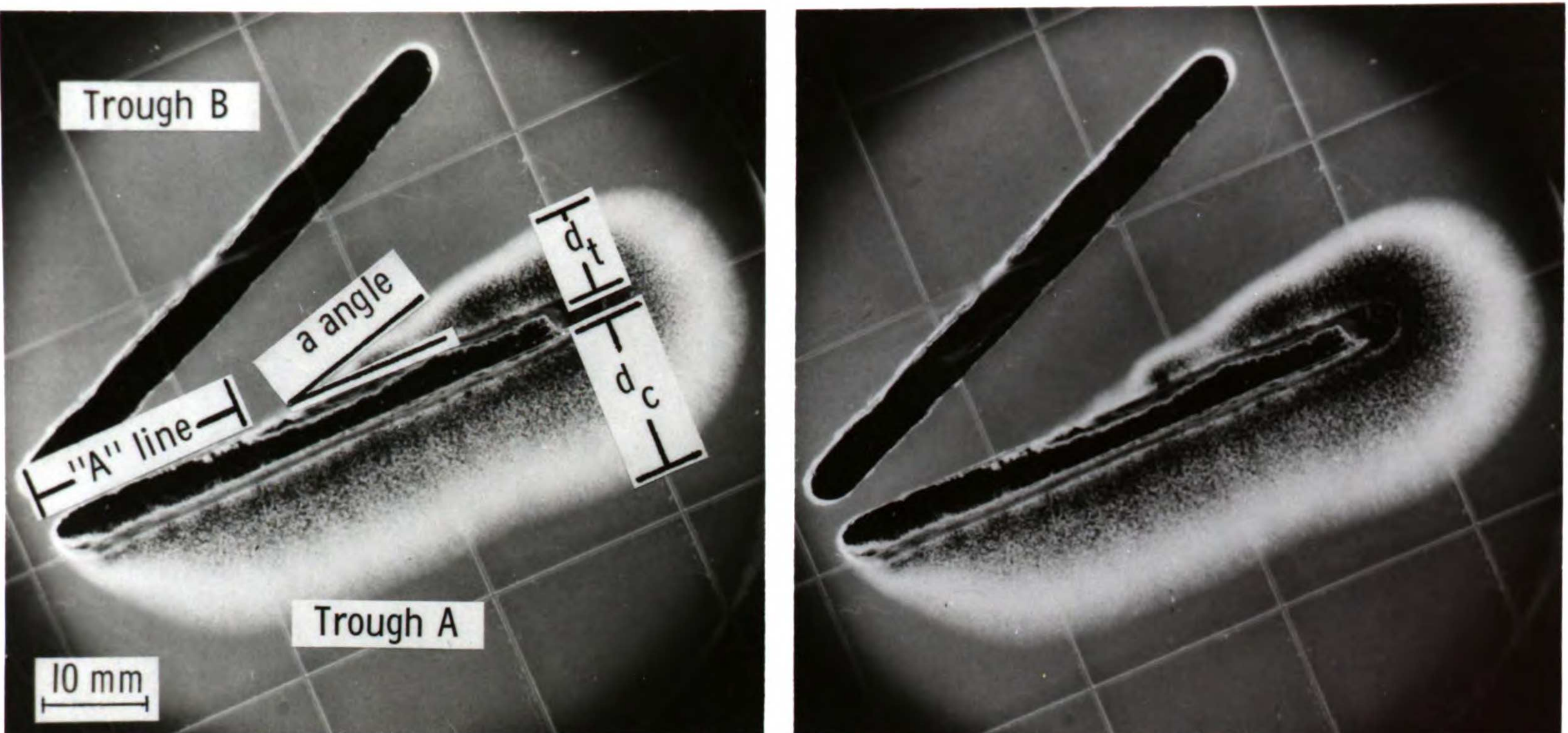

Figure 5. Growth inhibition pattern of _Aspergillus fumigatus_ to miconazole by the angle diffusion method (ADM).

the growing hyphal tips were exposed to decreasing concentrations of drug along the agar edge of the inoculum trough. This resulted in a growth pattern shown in Figure 5.

The 25° angle was selected experimentally because it gave the maximum inhibition zones with the instrument. Under standardized conditions, (0.2 ml of 100 µg/ml MCZ in trough B, 0.1 ml of 10^5 to 10^6 conidia or germlings/ml in trough A, 24 hours prediffusion at 4° C, 48 hours incubation at 35° C) at the 25° angle, inhibition of both spore and mycelial phases were greater than at larger angles. A lesser angle was impractical as will be discussed later.

Method of Analysis

The MIC value by the broth dilution method was defined as the lowest concentration of drug in SAB which inhibited all visible growth for 48 hours. In this time period, all strains grew a dense mat of mycelia in drug-free control broths. MIC values by the agar diffusion method were determined graphically (Figure 6) by the method of Cooper (49). Zones of inhibition were plotted against drug dose and a line of best fit (by the method of least squares) was extrapolated to the 8 mm zero point to determine the MIC.

Inhibition patterns obtained by the ADM method were expressed by three measurements:

1) The inhibtion of spore germination was referred to as the "A" line value. It was defined as the zone of complete absence of growth along the inoculum trough from the proximal end to the point where mycelial growth first appears.

2) The angle of growth formed from germinated spores tangent

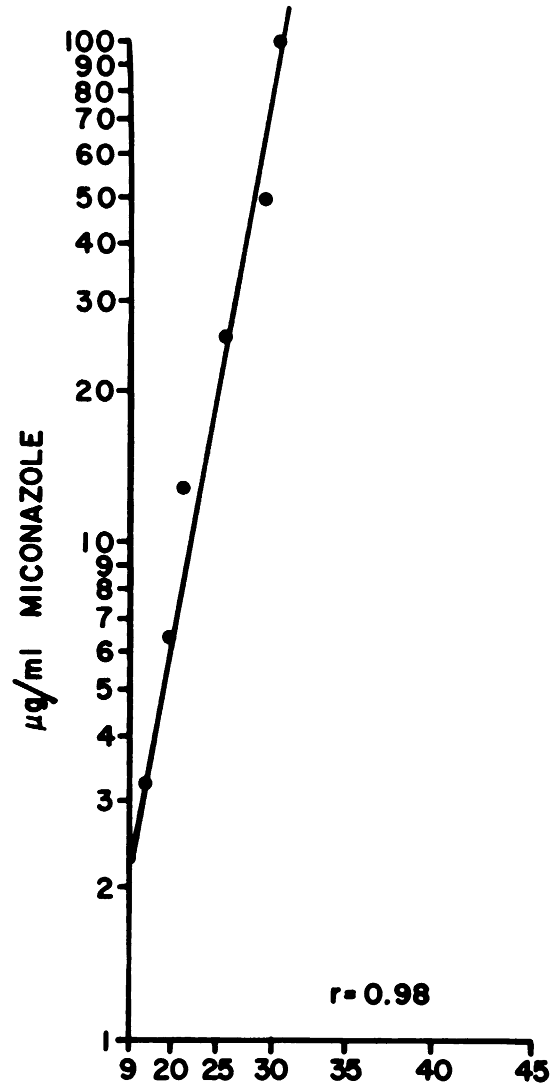

Figure 6. Inhibition of *A. fumigatus* germlings, strain 13, to miconazole by the agar diffusion method.

to the inoculum trough was referred to as the α angle.

3) The width of mycelial growth farthest from the drug trough but on the side adjacent to the drug trough was referred to as the d-test (d_t) value. The width of mycelial growth at the corresponding point but on the opposite side of the inoculum trough was referred to as the d-control (d_c) value. The ratio of the two, (d_t/d_c), was a measure of the inhibition of mycelial growth.

Each of these parameters was examined for possible correlation with broth dilution MIC values and agar diffusion MIC values. Data were analyzed by linear regression; the correlation coefficients were calculated and expressed as r values (50, 51). The significance of differences in MIC values to MCZ for spores and germlings of all strains of _A_. _fumigatus_ as tested by broth dilution and agar diffusion methods was determined by the ranking test of Mann Whitney (52, 53).

All inhibition tests were repeated at least three times; mean values and standard deviations were calculated to ascertain reproducibility.

RESULTS

Background Findings

No established reference method is available for evaluating a new technique, such as the angle diffusion method (ADM). In lieu, two published methods (26, 29, 35) frequently used for determining the inhibitory activity of antifungal drugs were selected and used in parallel with investigations on ADM.

The primary fungus under investigation in this study was *A. fumigatus* but certain studies were performed with *C. immitis*. The selection of these two fungi was based upon their recognized importance in public health and the selection was also influenced by the choice of MCZ as the test drug. *C. immitis* has been described (32) as highly susceptible to the drug's antimycotic action whereas *A. fumigatus* is considered moderately resistant, although some *Aspergillus* infections are still amenable to treatment with MCZ (20).

In initial growth studies, three media, Czapek, glucose yeast extract (1% glucose, 0.5% Difco yeast extract) (GYE) and SAB agar were compared. Growth of aspergilli was slow on Czapek medium but luxuriant on SAB and GYE in 48 hours. SAB was selected for several reasons: it was desirable, as earlier workers have pointed out (29), that the fungus be given every opportunity to grow well under test conditions, that prolonged growth should be avoided especially at temperatures favoring antimicrobic deterioration, MCZ tolerates an acid pH, such as SAB and, finally, SAB is widely used in mycological studies.

It became apparent during the study that different lots of SAB gave divergent inhibition results. MIC values obtained with 2 lots showed statistically significant differences by the Mann Whitney U

test at the 99% confidence level. Therefore all drug susceptibility comparisons were conducted using a single lot of SAB.

Early in the study, inocula were standardized and quantitated by spectrophotometer, plate counts and hemocytometer. The spore suspensions remained dispersed and cell viability was stable. The germinating cells, in contrast, tended to clump together, despite the use of the dispersing agent Triton X-100 and frequent vortex mixing; clumping decreased the initial colony forming unit counts by approximately one logarithm. Microscopic observations of germinated cells in wet mounts, by phase contrast, showed germination was not synchronous, in 8 hours of incubation at 35° C. Germinated conidial suspensions contained cells in various stages of development (Figure 2). They contained ungerminated spores, a predominance of swollen, irregular pear shaped spores, germinating cells with single germ tubes and, occasionally, germinated cells with multiple hyphal extensions. In order to limit further development of germinating cells into mycelial colonies, cell suspensions were refrigerated at 4° C.

Preliminary studies on the influence of the inoculum size upon MIC values showed that at the lower counts of 10^3 and 10^4 spores/ml, a lower MIC, 1.6 μg/ml, was observed at 48 hours but increased to 3.2 μg/ml at 96 hours. The suspensions containing 10^5 and 10^6 spores/ml gave an MIC of 3.2 μg/ml at 48 hours and remained stable at 96 hours. The effect of inoculum size was that the lower spore concentrations gave lower MIC values but was also dependent upon length of incubation time. Kitahara and Seth (54) observed a similar effect of inoculum size on MIC values with _Aspergillus_.

In all drug susceptibility tests the intravenous formulation of MCZ in Cremophor EL was used. Controls containing Cremophor carrier alone had no antifungal activity.

Spectrum of Susceptibility

The susceptibility profiles of all the test cultures are summarized in Tables 2 and 3. Clearly, *A. fumigatus* strains were inhibited by MCZ concentrations that were 9- to 22-fold higher, by broth dilution and agar diffusion methods, than those concentrations inhibitory to *C. immitis*.

The distribution of MIC values for the aspergilli by different methods is shown in Figures 7 and 8. The MIC values for *Aspergillus* spores in broth ranged from 1.6 to 16.8 μg/ml. There was overlapping with the corresponding MIC values for germlings which ranged from 2.1 to 25.2 μg/ml but, as will be shown, there was a significant displacement of the response spectrum between spores and germlings. In general, MIC values for spores, as determined by the agar diffusion method, were slightly higher than those obtained by broth dilution, but there was not as clear a distinction between germlings and spores by the agar diffusion method.

Results of MIC values for *Aspergillus* spores and germlings are summarized as follows:

1) For spores, by the broth method, 21 of 22 strains (95%) demonstrated MIC values of 6.4 μg/ml or less. One strain, *A. fumigatus* strain 8, had an MIC value of 16.8 μg/ml. This strain consistently showed the highest resistance to MCZ using either spores or germlings and by both assay methods. By the agar diffusion method, 9 strains (41%)

Table 2. Summary of Drug Susceptibility Values (Miconazole)

Organism (strain)	Minimum Inhibitory Concentration (MIC) (Standard Deviation in Parethesis)			
	Spore		Germling	
	Broth	Agar	Broth	Agar
A. fumigatus				
1	3.2 (0.0)	6.0 (3.1)	4.3 (0.6)	5.1 (3.2)
2	3.2 (0.0)	7.1 (3.1)	4.3 (0.6)	5.4 (2.0)
3	5.6 (0.7)	7.3 (2.3)	12.8 (0.0)	7.8 (1.2)
4	4.3 (0.6)	5.4 (0.4)	10.7 (0.6)	4.4 (0.8)
5	3.2 (0.0)	7.1 (1.2)	6.4 (0.0)	6.9 (1.8)
6	3.2 (0.0)	7.3 (2.0)	12.8 (0.0)	6.7 (0.1)
7	2.7 (0.4)	8.1 (2.9)	6.4 (0.0)	6.7 (2.0)
8	16.8 (0.6)	11.8 (5.9)	25.2 (1.2)	12.4 (7.2)
9	3.2 (0.0)	6.1 (2.3)	4.3 (0.6)	5.0 (0.7)
10	3.2 (0.0)	6.6 (1.7)	5.3 (0.6)	5.0 (1.5)
11	1.6 (0.0)	4.5 (0.3)	2.1 (0.6)	5.5 (2.9)
12	4.3 (0.6)	11.0 (2.9)	6.4 (0.0)	9.6 (1.7)
13	2.1 (0.6)	6.6 (2.9)	2.1 (0.6)	2.2 (1.6)
14	3.2 (0.0)	5.8 (2.5)	4.3 (0.6)	7.1 (2.9)
15	2.1 (0.6)	6.8 (2.6)	2.1 (0.6)	2.2 (1.4)
16	3.2 (0.0)	4.4 (1.4)	2.7 (0.6)	2.8 (1.9)
17	3.2 (0.0)	2.4 (0.7)	4.3 (0.6)	3.3 (1.1)
18	3.2 (0.0)	5.4 (0.1)	8.5 (0.6)	4.9 (1.4)
19	5.3 (0.6)	6.0 (1.0)	10.7 (0.6)	4.4 (0.9)
20	3.2 (0.0)	4.5 (0.5)	6.4 (0.0)	3.5 (0.8)
21	4.3 (0.6)	7.7 (1.3)	5.3 (0.6)	5.2 (2.0)
22	4.3 (0.6)	10.0 (4.2)	12.8 (0.0)	6.7 (0.8)
C. immitis				
Silveira (AS)	0.4	0.5		
Silveira (ES)	0.2	-		
46 (AS)	0.8	0.2		
46 (ES)	0.2	-		
Holl. (AS)	0.8	0.2		
Alex. (AS)	0.2	0.4		

Table 3. Summary of Drug Susceptibility Values (Miconazole)

Organism (Strain)	Angle Diffusion Method (ADM) 100 µg/ml (Standard Deviation in Parentheses)			
	Spore "A" line value		Mycelium d_t/d_c value	
A. fumigatus				
1	26.0	(1.0)	0.55	(0.06)
2	26.3	(2.1)	0.59	(0.04)
3	24.6	(1.2)	0.62	(0.05)
4	27.3	(0.6)	0.59	(0.04)
5	25.6	(2.5)	0.62	(0.01)
6	27.0	(1.7)	0.60	(0.03)
7	29.0	(3.6)	0.51	(0.02)
8	25.3	(4.2)	0.56	(0.06)
9	30.7	(4.0)	0.57	(0.09)
10	27.3	(0.6)	0.48	(0.09)
11	31.3	(3.0)	0.65	(0.08)
12	26.0	(1.5)	0.60	(0.06)
13	30.3	(0.6)	0.51	(0.06)
14	29.6	(0.6)	0.53	(0.00)
15	29.3	(1.2)	0.59	(0.02)
16	28.7	(1.2)	0.59	(0.02)
17	31.0	(1.7)	0.67	(0.07)
18	26.0	(3.5)	0.68	(0.04)
19	25.6	(1.2)	0.65	(0.07)
20	26.3	(0.6)	0.65	(0.04)
21	24.0	(1.7)	0.64	(0.00)
22	25.3	(3.8)	0.53	(0.00)

Table 3 Cont'd.

Organism (Strain)	Angle Diffusion Method (ADM) 5 μg/ml (Standard Deviation in Parentheses)			
	Spore		Mycelium	
	"A" line value		d_t/d_c value	
C. immitis				
Silveira	31.5	(0.7)	0.44	(0.30)
46	35.0	(1.4)	0.45	(0.00)
Holl.	23.5	(0.7)	0.32	(0.02)
Alex.	44.0	(1.4)	0.52	(0.03)

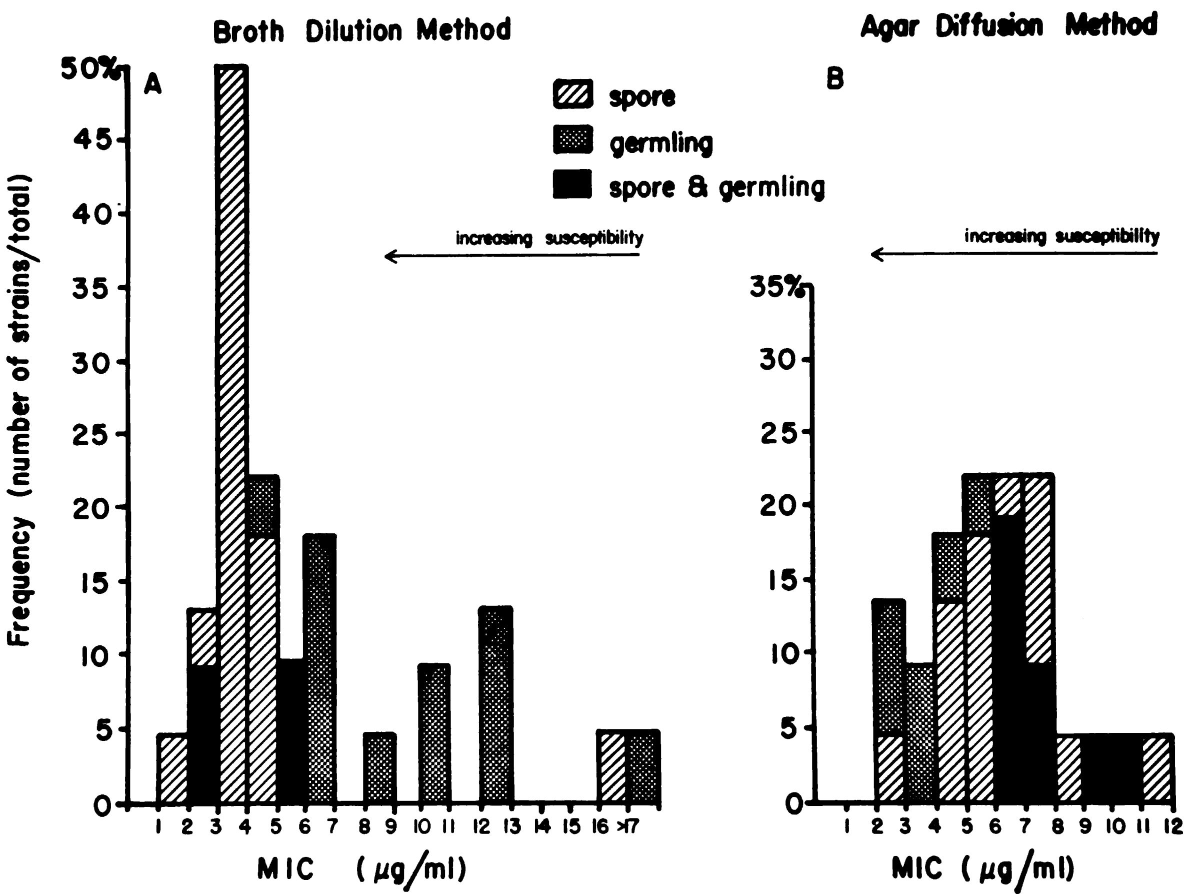

Figure 7. Distribution of spore and germling MIC values by broth dilution (A) and agar diffusion (B) methods for 22 strains of _A_. _fumigatus_ to miconazole.

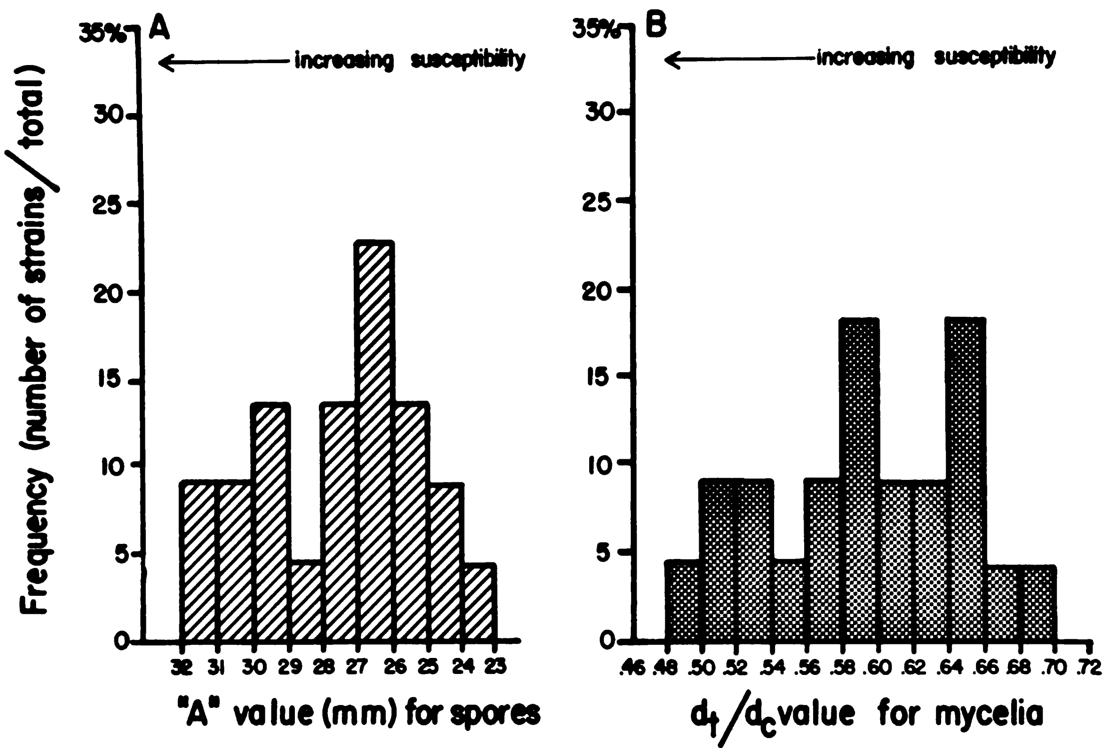

Figure 8. Distribution of spore (A) and mycelial (B) inhibition values by angle diffusion method (ADM) for 22 strains of A. fumigatus to miconazole.

were susceptible to 6.4 μg/ml or less, 20 strains (91%) had MIC values of 10 μg/ml or less and 2 strains (9%) were susceptible to 11-12 μg/ml.

2) For germlings, by the broth method, 15 strains (68%) were susceptible to 6.4 μg/ml or less, 21 strains (95%) had MIC values of 12.8 μg/ml or less and one strain (5%), strain 8, was susceptible to 25.2 μg/ml. By the agar diffusion method, 13 strains (64%) demonstrated MIC values of 6.4 μg/ml or less and 21 strains (95%) had values of 10 μg/ml or less. The resistant strain 8 had an MIC of 12.4 μg/ml.

There is general agreement between results presented here and the findings of Shadomy and Paxton (55) who, using a different method, reported that 5 strains of _A. fumigatus_ showed MIC values in excess of 4 μg/ml.

The susceptibility of the endospore and arthrospore phases of _C. immitis_ (2 strains) (Table 2) by broth dilution and agar diffusion methods are in agreement with results published previously (21, 34).

In its present state, precise quantitation of susceptibility values by the ADM is not possible and therefore comparison and correlation between it and other methods is not proposed. Nevertheless, clear distinction in the relative susceptibilities of _A. fumigatus_ and _C. immitis_ using this method was seen. In order to generate similar ADM "A" line and d_t/d_c values, MCZ had to be used at 1/20th the concentration (5.0 μg/ml in the drug trough) for _C. immitis_ than for the more resistant _A. fumigatus_.

The numerical values for the "A" line (spore inhibition) and the

d_t/d_c value (mycelial inhibition) should not be compared to one another for a given strain. But individual "A" line values can be compared among the different strains of *A. fumigatus* to assess the spread of response pattern. The distribution of spore and mycelial inhibition value by ADM is shown in Figure 8.

Broth Dilution Method

All strains of *Aspergillus* grew luxuriantly, to the point of sporulation, in 48 hours in the drug-free control broths. Broth dilution tests with *C. immitis* required 6 days of incubation for good growth to appear in control broths. The MIC profile of 22 *Aspergillus* strains may be summarized as follows: spores - mean 3.6 μg/ml, median 3.2 μg/ml, range 1.6 to 16.8 μg/ml. The corresponding data for germlings are: mean 5.9 μg/ml, median 5.3 μg/ml, range 2.1 to 25.2 μg/ml (Tables 2 and 3).

The difference in MIC values between spores and germlings was significant ($p<0.05$, Mann Whitney U Test). In 86% of 22 strains, the germlings were more resistant to MCZ than spores by a mean factor of 1.8 and a range of 0.8 - 4.0 μg/ml. The relationship between spore and germling MIC values is presented in Figure 9. The regression line gave a correlation coefficient ($r = 0.87$), suggesting a correlation despite the displacement in MIC values.

Agar Diffusion Method

Zones of inhibition were measured without magnification using an illumination box. Frequently with germlings, and especially at the lower drug dilutions of 1.6 μg/ml to 12.8 μg/ml, resistant colonies appeared within the zone of inhibition or at the edges giving the zones imprecise margins. In these cases, an average zone diameter was

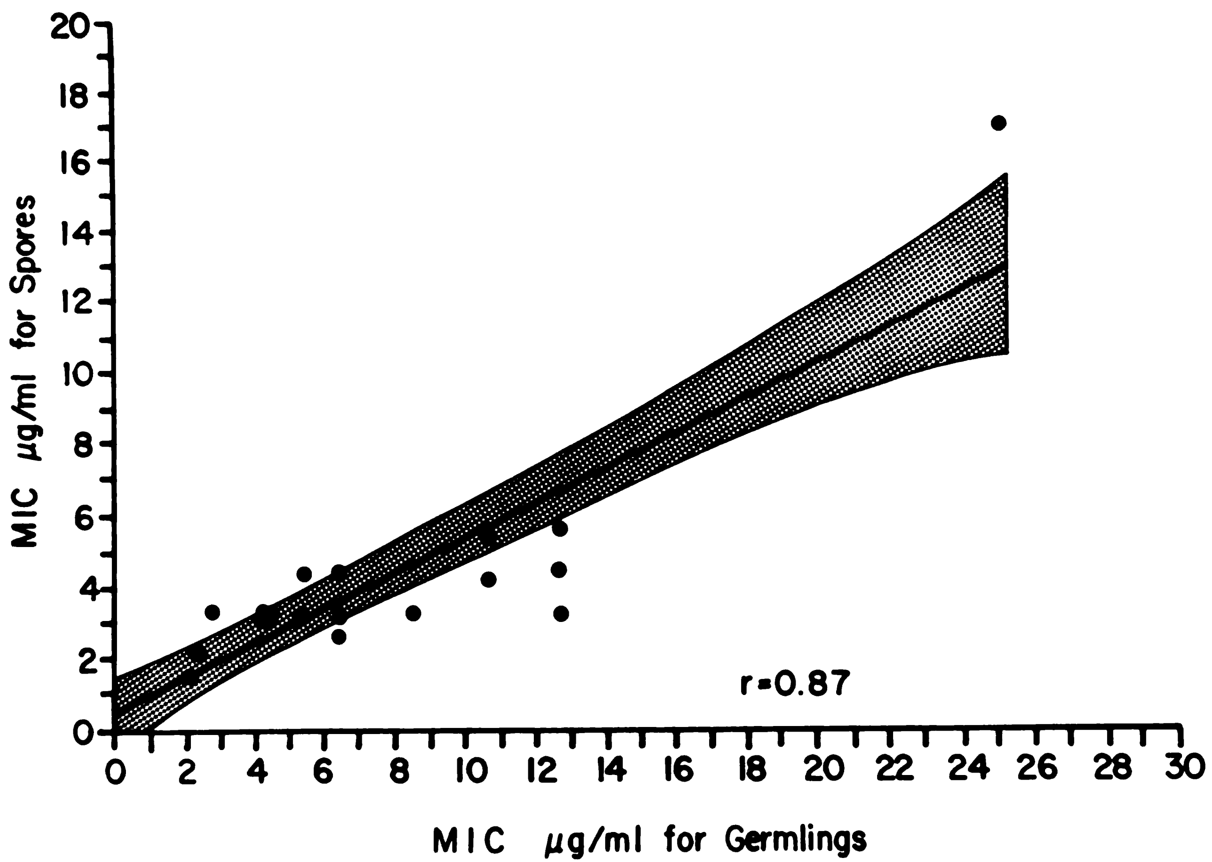

Figure 9. Relationship of MIC values of spores and germlings by broth dilution method for 22 strains of _A_. _fumigatus_ to miconazole; shaded area = 95% confidence limits of line of best fit for all points.

obtained from multiple measurements. If the colonies appeared within the zones as pinpoint and few in number, they were disregarded. If they were large and confluent, no zone was measured. Spore inocula frequently produced sharper zones than germlings.

Many strains of aspergilli grew luxuriantly, frequently producing powdery blue-green, tan, grey and brown sporulation on the agar surface in 48 hours. A few strains produced only white mycelia on test plates from both spore and germling inocula. Hence, strain variation associated with lack of sporulation, spore pigmentation and growth rate was evident.

The agar diffusion MIC data (Figure 7B) is summarized as follows: spores - mean 6.7 μg/ml, median 6.6 μg/ml, range 2.4 μg/ml to 11.8 μg/ml. The corresponding data for germlings are: mean 5.6 μg/ml, median 5.1 μg/ml, range 2.2 μg/ml to 10.1 μg/ml. Spores appeared somewhat more resistant than germlings but this was not statistically significant at the 90% confidence interval. In contrast, significant differences did occur by the broth method (Figures 7A and 9). Further, the MIC range determined by the broth dilution method was broader than the range observed by agar diffusion (Figures 7A and 7B).

The relationship between MIC values of spores and germlings by the agar diffusion method is presented in Figure 10. The correlation coefficient ($r = 0.76$) suggests only a loose relationship.

ADM

Since the ADM method is new, it is necessary at this point to indicate briefly the conceptual basis for the different measurements. Further discussion will be presented in the next section.

1) The "A" line value represents a zone of inhibition

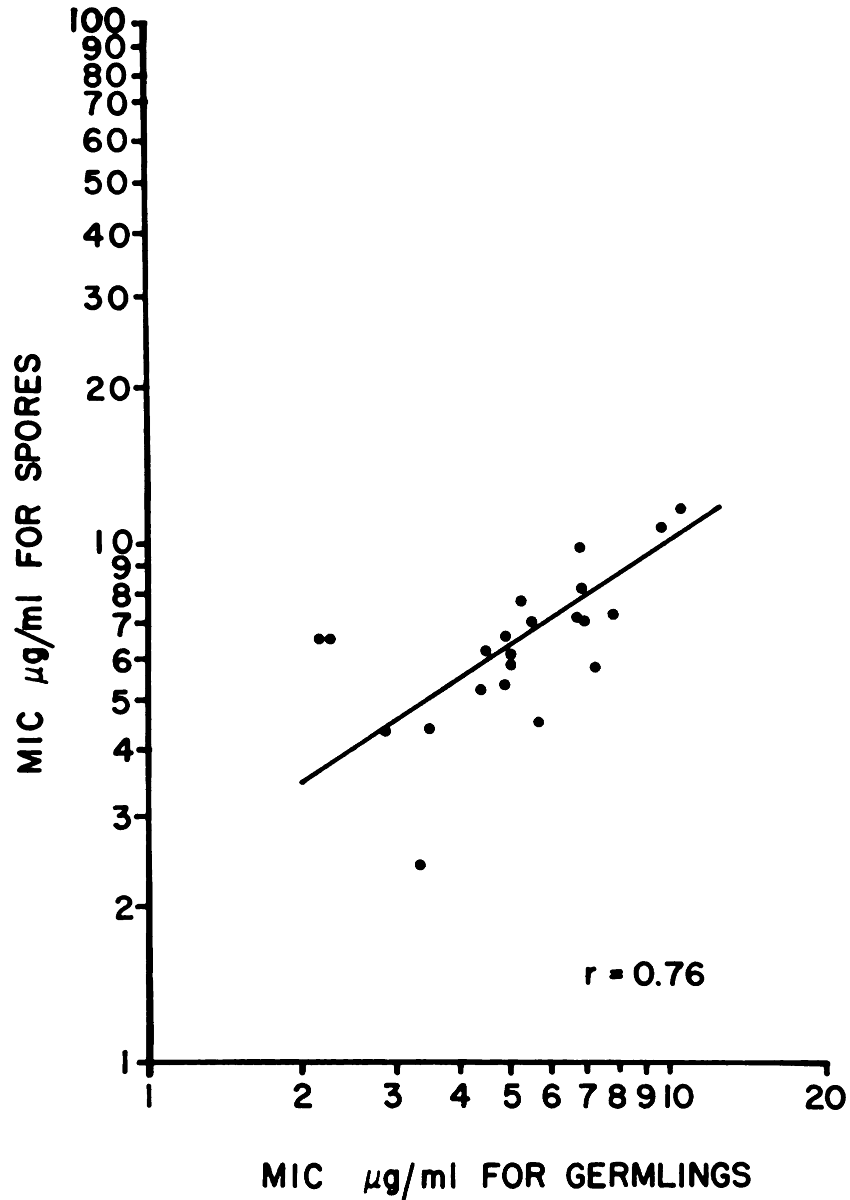

Figure 10. Relationship of MIC values for spores and germlings by agar diffusion method for 22 strains of A. fumigatus to miconazole.

of spore germination, determined by the drug concentration gradient formed by the diffusion of drug through the agar. An increasing "A" value generally indicates increasing spore susceptibility to the drug being assayed.

2) The d_t/d_c value reflects the inhibitory response of the growing hyphal tips to the drug. The lower the value, the greater the susceptibility to the drug being studied. An important factor influencing the value is the mycelial growth rate expressed by the d_c portion of the value (Figure 5). If the d_c value is small, as seen in the slower growing strains, the significance of the d_t value diminishes; conversely, a relatively small d_t value with a relatively large d_c value increases the significance of the d_t inhibition.

3) The α angle is another measurement of mycelial inhibition (Figure 5). Its magnitude is affected by the growth rate of the strain and by the diffusion rate of the drug approaching the growing hyphal tips.

In early studies with ADM, the mycelial growth at the α angle zone was viewed microscopically for any aberrant hyphal morphology, in response to MCZ, as had been seen with the antimycotic drug Griseofulvin (56). However, none was observed when compared to the control growth.

The relationship of the ADM "A" values to agar diffusion spore and germling MIC values is presented in Figure 11. The correlation between "A" line values and agar diffusion MIC values ($r = 0.82$) for spores was good (Figure 11). This was expected since both methods measured spore susceptibility at a single comparable drug concentration (100 µg/ml in both methods). Similarly, as expected, the relationship was less close

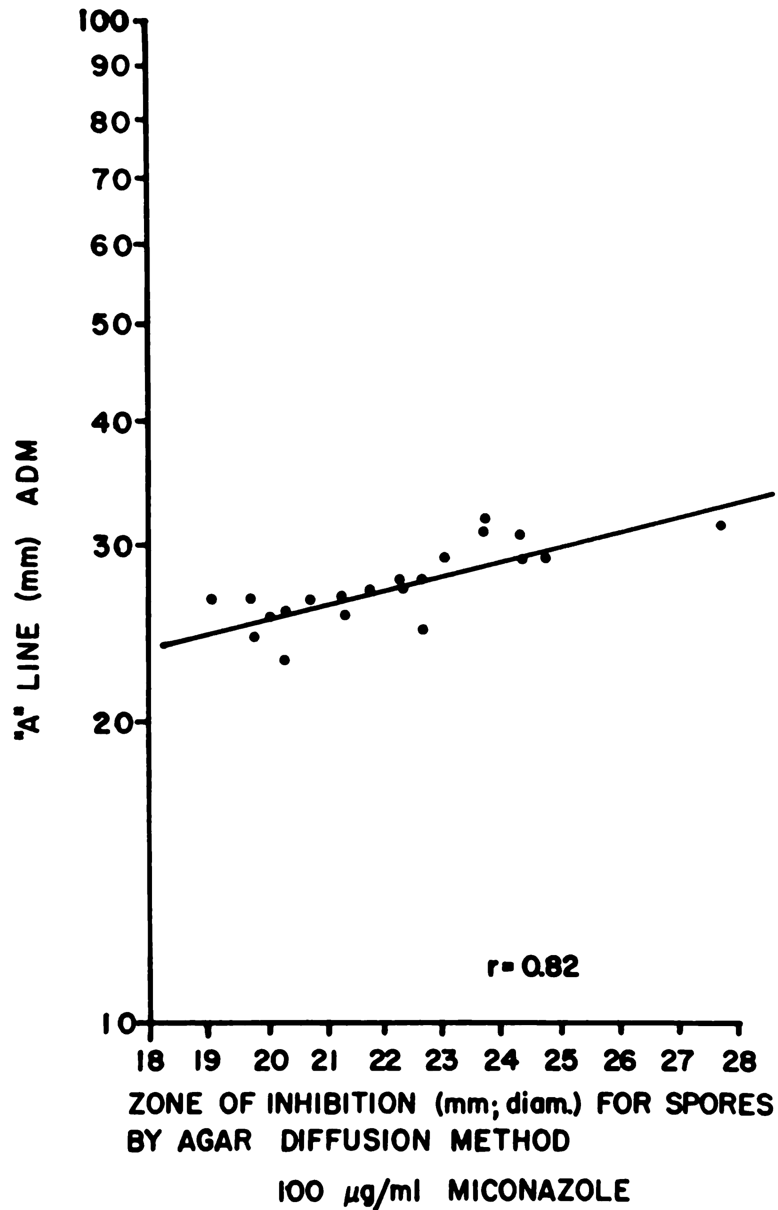

Figure 11. Relationship of spore inhibition values measured by angle diffusion method (ADM) and by agar diffusion (constant drug concentration) for 22 strains of A. fumigatus.

(r = 0.64) (Figure 12) when the "A" value for spores was compared to the MIC value for germlings. Also, as expected, using the ADM method alone, the "A" value for spore susceptibility showed poor relation with the d_t/d_c value (r = 0.1) for mycelial susceptibility (Figure 13). Unexpectedly, however, the α angle correlated poorly (r = 0.43) (Figure 14) with the d_t/d_c value. This may reflect the fact that the α angle value alone, unlike the d_t/d_c ratio, provides no accommodation for the growth rate factor of individual strains.

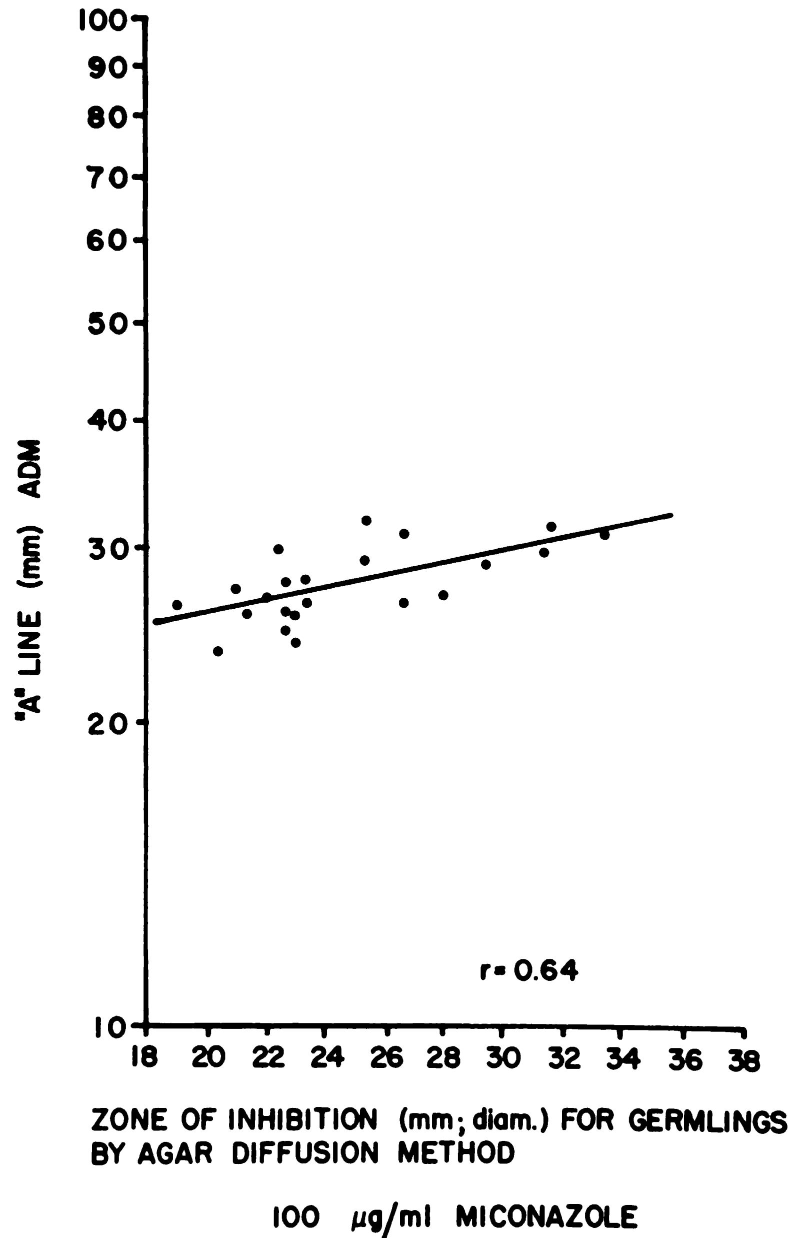

Figure 12. Relationship of inhibition values of spores and germlings by angle diffusion method (ADM) and agar diffusion (constant drug concentration) for 22 strains of A. *fumigatus*.

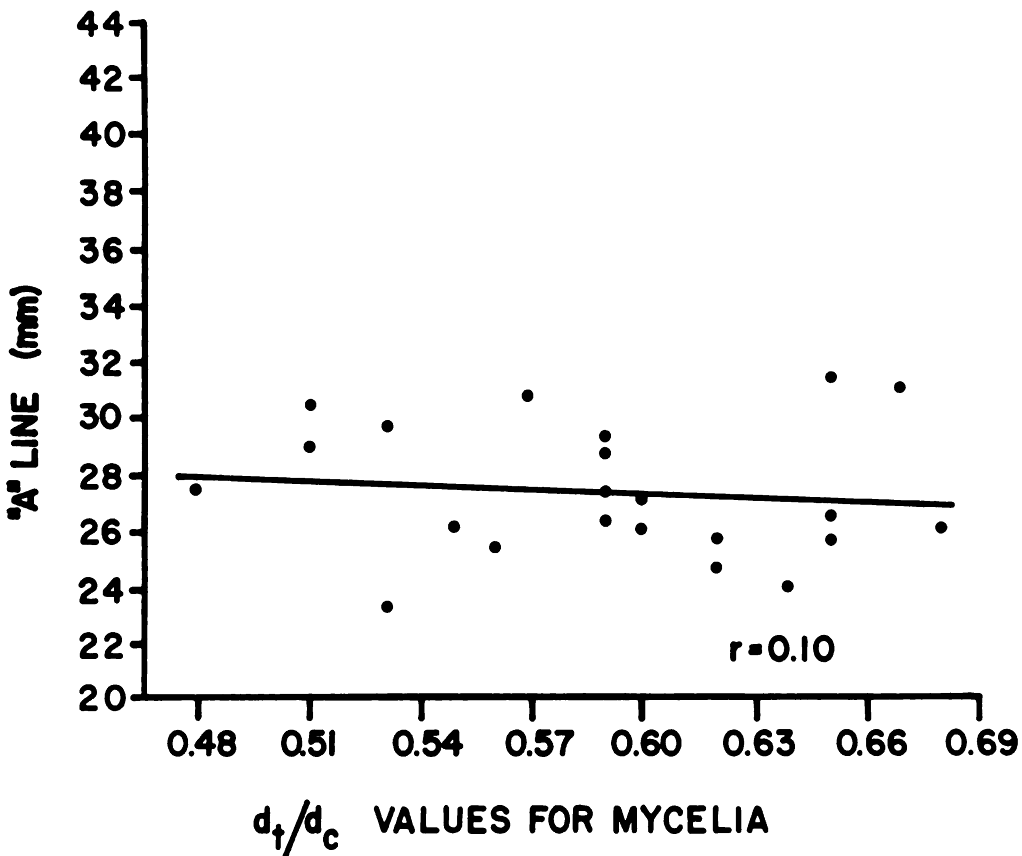

Figure 13. Lack of correlation between spore and mycelial inhibition values measured by angle diffusion method (ADM) for 22 strains of *A. fumigatus* to miconazole.

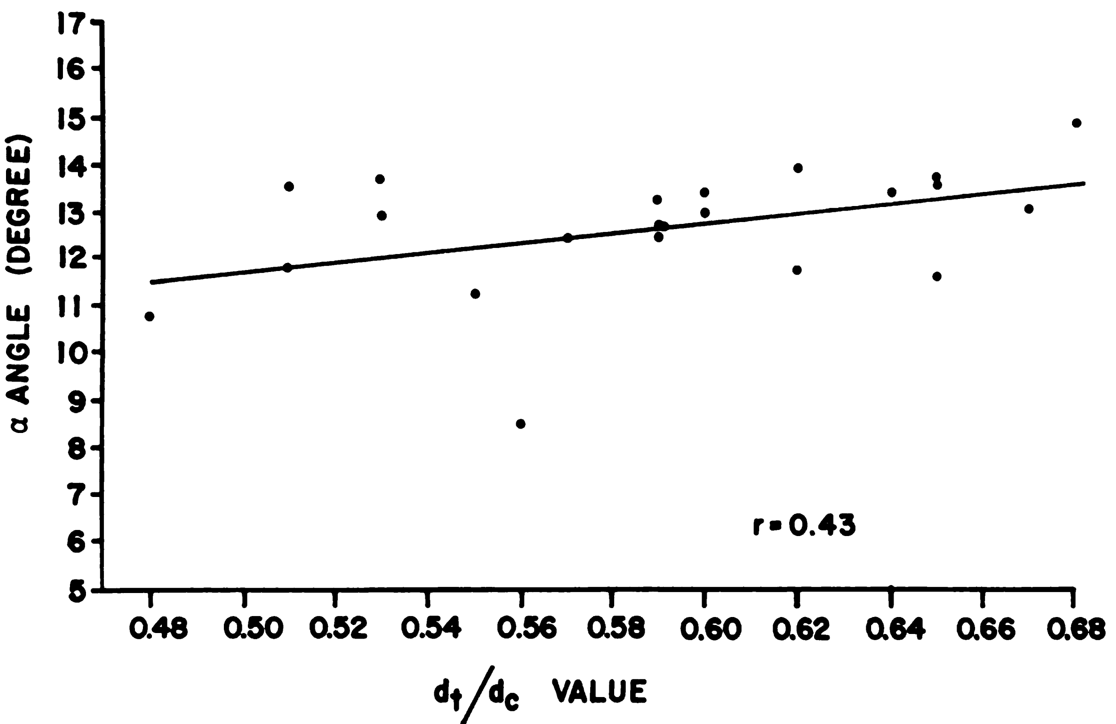

Figure 14. Relationship of mycelial inhibition values measured by angle diffusion method (ADM) for 22 strains of _A. fumigatus_ to miconazole.

DISCUSSION

Currently, there are no standardized antifungal drug susceptibility methods. Furthermore, variables occur in fungal susceptibility tests that have not been well studied. They include the choice of medium, duration and temperature of incubation, use of the appropriate morphological growth phase of the fungus and finally, interpretation of the inhibition values in terms of relative susceptibility of the fungus to drugs in vivo (29, 41, 42).

Accordingly, the objectives of this study were 2-fold: first, to determine whether the drug susceptibility profiles of conidia and germlings of *A. fumigatus* differed using published methods, and secondly, to develop a drug susceptibility method that allowed simultaneous visualization of the relative susceptibilities of spores and growing hyphal tips.

Comparisons of the drug susceptibility profiles of conidia and germlings by broth dilution and agar diffusion methods indicated that the MIC value was influenced by both the morphological form of the fungus used as well as the type of assay method employed (Table 2 and Figure 7). The influence of the two assay methods was seen on the mean MIC values for spores: 3.6 μg/ml by broth dilution method and 6.7 μg/ml by agar diffusion method. Germlings, however, were less affected by the assay method and exhibited a negligible discrepancy between mean MIC values, 5.9 μg/ml by broth dilution and 5.6 μg/ml by agar diffusion (Figure 7). These results are in general agreement with results obtained by Shadomy and Paxton (55) and Schär and Kayser (57) although the morphological form of their inocula was not specified.

Differences between MIC values for spores and germlings may reflect in part the mode of action of MCZ and also the influence of the analytical method used to obtain the MIC value. As mentioned previously, MCZ inhibits both the cytochrome c peroxidase and catalase enzymes and the development of cellular, mitochondrial and vacuolar membranes. There are resulting changes in the function and permeability of the membranes (16, 17, 18, 19, 58). For fungistasis to occur, sufficient drug must enter the cell and remain active in its inhibitory function. Studies on dormant conidia reveal they contain fewer and smaller mitochondria, nuclei and vacuoles than the parent mycelium (59). Germinating conidia, in contrast, have a greater cell membrane surface area associated with their increased cell size (60). Germlings are also metabolically more active and contain increased number of organelles in preparation for forming new hyphal extensions (60). It is possible, therefore, that a higher level of MCZ is required to inhibit germlings than conidia based upon the numerous metabolically active membranes and greater enzymatic activity of germlings.

Another possible explanation of the difference observed between conidia and germling MIC values is related to spore germination time. A. fumigatus conidia have an approximate 8 hour lag time at 35° C; during this time, spores develop internally in preparation for germination (60). The germlings, on the other hand, initiate hyphal growth quickly at 35° C. Germlings, therefore, have a metabolic head start over spores and may be capable of temporarily overcoming the effects of the drug until a higher fungistatic level is achieved.

Certain limitations in the broth dilution method should be mentioned. The accuracy of the endpoint can be read only to one tube

dilution. Thus, an MIC endpoint of 6.4 μg/ml, for example, is more accurately expressed as >3.2 μg/ml but ≤6.4 μg/ml; precise MIC values by this method are not readily achieved.

Somewhat more precision may be obtained by the agar diffusion method. A major difference between the broth dilution and agar diffusion methods is that the MCZ concentration in the broth is uniform; a single drug concentration acts on all fungal cells in the tube simultaneously. In the agar diffusion method, a continuously falling drug concentration gradient in the agar occurs, from the center of the cup outward (61). As the drug concentration changes with time, germinated spores grow towards the cup until an inhibitory concentration is reached. Therefore, the reading time of the zone of inhibition was critical. In this study, smaller zones were indeed observed after 48 hours.

Another limitation of the agar diffusion method is related to the calculation of the MIC value (62, 63). Many strains exhibited a sigmoidal response curve when the diameters of the zones of inhibition were plotted against the logarithm of the drug concentration (Figure 6). A flattening of the curve occurred at the lower drug concentrations. This effect is disregarded by extrapolating from the line of best fit to the zero point to obtain the MIC value. In effect, this procedure gives a lower MIC value than that obtained by extrapolating directly from the points at the lower end of the curve.

Somewhat minor limitations of the agar diffusion method are that it takes 24 hours longer to obtain results compared to the broth dilution method and it requires numerous critical technical manipulations. These include obtaining agar of uniform depth, drying of the agar surface,

overlaying the molten agar inoculum to obtain even distribution of cells and accurate dispensing of drug solutions into cups (64). These manipulations also increase the chance of airborne contamination.

The agar diffusion method, however, has certain advantages: there is a continuous drug concentration which allows the visualization of resistant colonies within the zone of inhibition. This gives important information on the occurrence of a resistant subpopulation. The reproducibility of this method was better than the broth method. The coefficient of variation for agar diffusion MIC values, between the three independent trials, did not exceed 58% whereas with the broth dilution method, the maximum coefficient of variation was 84%.
Broth dilution endpoints were generally reproducible within one doubling dilution.

The third method described in this study, the angle diffusion method (ADM), was designed as a research tool for observing the effects of an antifungal drug on actively growing hyphal tips. The importance of the ADM methodology does not lie in the closeness of the correlation of the "A" line measurement and the d_t/d_c measurements with the conidial and mycelial MIC values obtained by the other two methods. It is, rather, a technique enabling one to: 1) make inhibition measurements simultaneously and on the same growing culture and 2) give a quick visual index of relative susceptibilities of the conidial and mycelial forms. Thus, a large "A" line value and a high d_t/d_c value, or ratio, indicates relative mycelial resistance and conidial susceptibility. A low "A" value and low d_t/d_c ratio would indicate relative mycelial susceptibility and conidial resistance.

The technique of employing steeply falling concentration gradients in experimental biological systems was used by Osgood (65) in 1955 for growing blood cells in tissue culture. By simply inserting a sterile slide at a 45° angle into a deep suspension of cultured cells, he was able to obtain a continuous gradient of oxygen tension. More recently, with antimicrobics, Barry (66) describes angular diffusion patterns in agar using 2 troughs for the purposes of demonstrating antagonism or synergy between two drugs.

The ADM technique provides a more steeply falling drug concentration gradient than conventional agar diffusion methods employing discs or cups. This is due to the drug diffusing at an angle (25°) relative to the even front of mycelial growth. The resultant growth pattern reflects both the inhibition of germination of conidia and inhibition of growing hyphal tips.

The advantages of the ADM method as a qualitative screening test for filamentous fungi are: 1) a single drug concentration is used for determining the relative susceptibility to a drug; 2) the growth rate of the fungus is observed unaffected by drug within the same test; this serves as a control on growth conditions; 3) the influence of the drug is seen on two morphological stages of the fungus, the germinating spore and growing hyphal tips within the same test; 5) inhibition values are quickly visualized and measured directly. Numerous drugs could be assayed conveniently to ascertain which was the most efficacious for the morphologic phase of interest. The complex geometry of the ADM test system and the diffusion dynamics of the drug are not well understood and need additional investigation. In addition, at this time, some technical problems with the ADM still exist. The

temperature of the agar in the plate, prior to cutting the troughs, is critical. Cutting the troughs when the agar was at room temperature alleviated the problem of agar splitting at the narrow agar bridge between troughs at the proximal end. Regulating the amount of vacuum pressure required to remove the agar from the troughs, so as not to crack the surrounding agar, remains a problem. The size of the angle between troughs was limited to 25° because, with the instrument as designed, a lesser angle would break the agar bridge at the closest point between troughs. A narrower angle should increase the sensitivity of the method.

All of the in vitro drug susceptibility findings presented should be viewed from the perspective that the value of any inhibitory test lies in its relationship to therapeutic efficacy and to the pharmacokinetics of the drug in question. With MCZ, peak human serum concentrations in man may range from 2.3 to 7.5 μg/ml of active drug depending on intravenous dose levels (12). Since the half-life of MCZ in vivo is approximately 30 minutes, treatment has included frequent and prolonged administrations (15, 23).

Symoens (20) has described indices of MCZ susceptibility based upon peak serum levels attainable in patients as:

$\leq$ 0.1 μg/ml	very sensitive
1 - 10 μg/ml	sensitive
10 - 100 μg/ml	marginally sensitive
>100 μg/ml	not sensitive

The majority of strains (90%) tested in this study fall in the sensitive category based on results obtained with conidia by both broth dilution and agar diffusion methods. A higher percentage (30%) of

strains would be categorized as marginally sensitive based upon germling results using both of these methods. However, therapeutic outcomes cannot be strictly predicted by MIC value results alone. Other factors, outlined below, singly and together influence the drug's usefulness in disease.

No agar, broth or synthetic menstrum can replace the immensely complex system of diseased animal tissue. Important factors affecting therapeutic outcome are: 1) the capacity of the host's intricate immune system to respond to invading organisms, 2) metabolic alteration of the drug, its half-life and its toxicity, 3) the interaction of drugs with one another to produce antagonism or synergy, 4) the capacity of a drug to penetrate into a lesion or to overcome tissue barriers such as the blood-brain barrier and 5) the binding properties of the drug to plasma proteins or to tissue or humoral sites (67). Any one of these factors can mitigate against a drug's influence even on exquisitely susceptible microorganisms.

Attempts at precise quantitation of the in vitro activity of a drug may be of limited value to the clinician. Such results are influenced by a large number of variables as well as sources of error in presently available susceptibility tests (29, 64). Better correlation of in vitro results with clinical therapy is needed so that MIC values or other inhibition indices, such as the ADM, can be useful in clinical medicine.

An important factor of in vitro testing is the fungal inoculum, as already noted. This study indicates that the germling phase of _A. fumigatus_ is more resistant to MCZ than conidia. However, it is not possible to predict the relative susceptibility level of germlings by

the susceptibility of the conidia. These findings, in conjunction with the knowledge that growing hyphae are the invasive form within the diseased host, suggest that hyphal tips or germlings be employed for susceptibility tests rather than conidia (or a mixture of conidia and mycelia) as is now generally the case (13, 26, 29, 31, 41).

Current in vitro drug susceptibility methods have qualitative applications in determining a relative drug response level of a fungal isolate and in ascertaining if the organism changes in its susceptibility after exposure to a particular drug therapy. However, assignment resistance or susceptibility to an organism should be made in context of attainable drug levels in the host (64). An invitro method such as the ADM presented in this study has application in that it is rapid, inexpensive and gives data on the susceptibility of the different morphologic phases of fungi such as those of _A_. _fumigatus_. The ADM method can thus make a contribution to susceptibility testing as a screening test for antifungal agents against the increasing number of fungi involved in diseases of humans, animals and plants.

SUMMARY

Drug susceptibility profiles of both the conidial and hyphal morphologic forms of *Aspergillus fumigatus* were compared. Twenty-two strains were examined using miconazole (MCZ), an antimicrobic moderately antagonistic to the aspergilli.

Conventional agar diffusion and broth dilution methods were employed in determining susceptibility profiles. In addition, a new technique for determining susceptibility, the angle diffusion method (ADM), was developed as a research tool for observing the effects of antifungal drugs on actively growing hyphal tips. The ADM method permitted the relative susceptibilities of the conidial and hyphal forms of the fungus to MCZ to be visualized on a single agar test plate. The system employed troughs cut at an acute angle to each other. The diffusing drug from one trough interacted with growing advancing hyphal tips from the other. This produced a unique growth inhibition pattern which resulted from a steeply falling drug concentration gradient created by the angle between troughs. For purposes of comparison, angle diffusion studies were performed with *Coccidioides immitis*, an organism highly susceptible to the inhibitory action of MCZ.

The ADM method allows a visual index of relative susceptibilities of conidial and hyphal forms simultaneously. It has applications as a screening test for antifungal agents against numerous pathogenic and opportunistic fungi.

Comparisons were made of the relative susceptibilities of the different growth phases of *Aspergillus fumigatus* employing broth dilution and agar diffusion methods. Conidia from the majority of strains were relatively susceptible to MCZ and gave MIC values between 1-10 ug/ml. However, these values were significantly influenced by the assay method; higher values were given by the agar diffusion method than by the broth dilution method. In contrast, values for germlings were less influenced by assay methods and these structures were more resistant to MCZ than conidia as determined by either assay method.

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