Colorimetric Susceptibility Testing for Aspergillus fumigatus: Comparison of Menadione-Augmented 3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl-2H-Tetrazolium Bromide and Alamar Blue Tests

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Received 22 January 1996/Returned for modification 2 March 1996/Accepted 11 May 1996

Two colorimetric methods that use Alamar Blue or 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) for assaying the in vitro activities of antifungal agents have been described. We report that both tests performed similarly when the antifungal activity of amphotericin B against Candida albicans was determined. However, only the MTT test generated interpretable data when Aspergillus fumigatus was used.

Because conventional in vitro susceptibility testing of fungi is fraught with methodological pitfalls, the recent development of colorimetric tests based on measurements of metabolic activity is both welcome and promising. Alamar Blue is an oxidation-reduction indicator for eukaryotic cells, and its utility for assessing the viabilities of yeasts has been demonstrated (1, 8–10). A second approach, based on the detection of mitochondrial activity, was first described by Tellier et al. (12), who used 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide in a macrolidation assay for Candida albicans. An improved microtiter assay based on a menadione-augmented 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reaction was subsequently developed in our laboratory (5). The test appeared to be applicable to both C. albicans and Aspergillus fumigatus. Information on the utility of the Alamar Blue test for assessing the activities of antifungal agents against A. fumigatus is lacking, so a comparison of the two tests was undertaken in the present study.

C. albicans 0815 and 8166 were from our diagnostic laboratory. Strain R64, exhibiting a relative resistance to amphotericin B, was provided by H. Dermoumi, Institute of Microbiology, Essen, Germany, and strain 3059 was a gift from E. Liehl, Sandoz Institute, Vienna, Austria.

All strains have previously been used in the MTT test (5), and strain 0815 has been used in flow cytometry studies (6, 7). Preparation of yeast cells for colorimetric susceptibility testing was as described previously (5), and 10⁵ cells were seeded into each well of flat-bottom microtiter plates (Greiner, Nürtingen, Germany).

Eight A. fumigatus strains were obtained as clinical isolates from patients with Aspergillus pneumonia or invasive aspergillosis. Strain ATCC 46645 was supplied by A. Brakhage (Institute of Microbiology, Ludwigs-Maximilian-University, Munich, Germany). After subculture on Sabouraud agar, conidial suspensions were prepared as described by Rolides et al. (11). The conidial suspensions were filtered through a sterile 40-μm-pore-size nylon mesh (Falcon, Heidelberg, Germany). Penicillin (100 U/ml) and streptomycin (100 μg/ml) (antibiotic mix; Gibco, Karlsruhe, Germany) were added, and the suspensions were stored at 4°C. For susceptibility testing, 5 × 10⁴ conidia per well, determined by counting in a Neubauer chamber, were seeded into flat-bottom microtiter plates in 200 μl of tryptic soy broth, and the plates were incubated for 12 to 14 h at 30°C. This allowed for the hyphal outgrowth of more than 95% of the conidia. Amphotericin B was from Squibbs Pharma, Vienna, Austria, and was used as described previously (5). Macrodilution susceptibility testing was performed in morpholinepropanesulfonic acid (MOPS)-buffered RPMI (Sigma) according to the standard procedures for C. albicans of the National Committee for Clinical Laboratory Standards (4).

Dilutions of amphotericin B were prepared in 1 ml of RPMI, and 10³ C. albicans cells were inoculated into each tube. The turbidity was read visually after incubation for 48 h at 35°C. For microdilution colorimetric assays, 10³ yeast cells in 100 μl of RPMI were seeded into each well of flat-bottom microtiter plates, amphotericin B in 100 μl of RPMI was added, and incubation was continued for 24 h at 37°C.

For testing of A. fumigatus isolates, hypha-containing wells were washed twice in saline and were refilled with 100 μl RPMI. Amphotericin B (100 μl) was added, and the plates were incubated for 24 h at 30°C. MICs were calculated in comparison with those for controls and are given as the MICs at which 90% of isolates are inhibited (MIC₉₀).

Alamar Blue was from Labserv (Giessen, Germany), and the assay was performed as described previously (10). The results of triplicate assays were read in a Titertek Fluoroscan II system (excitation wavelength, 544 nm; emission wavelength, 590 nm). For development of the MTT test, 25 μl of RPMI containing 5 mg of MTT (Serva, Heidelberg, Germany) per ml and 1 mM menadione (Sigma) were added, and the mixture was incubated for 3 h at 37°C. Plates containing C. albicans isolates were then spun at 1,000 × g for 10 min, and the supernatants were carefully aspirated. Centrifugation was unnecessary for A. fumigatus isolates because the fungi adhered firmly to the plates. A total of 0.1 ml of acidic isopropanol (95 ml of isopropanol, 5 ml of 1 N HCl) was added to each well, and the plates were placed on a shaker for 5 min to dissolve the formazan crystals. Triplicate assays were performed in a Titertek Multiscan spectrophotometer at 550 nm.

A chemiluminescence assay modified from the assay of Yamashoji et al. (13–15) was used to test for surface oxidase. A total of 1 × 10⁵ C. albicans cells or 5 × 10⁴ A. fumigatus hyphae in microtiter plates were incubated in 250 μl of Dulbecco
modified Eagle medium (Biochrom, Berlin, Germany) containing 250 μM menadione for 30 min at 37°C to allow H₂O₂ production, and 50-μl aliquots were then transferred to white microwell strips (Greiner) for bioluminescence measurements (Luminometer LP96g; EGG Berthold, Bad Wildbad, Germany). Fifty microliters of oxalate ester [oxalic acid bis(2,4-dinitrophenyl)ester; 2 mg/ml; Sigma] and pyrene (0.4 mg/ml; Sigma) dissolved together in acetonitrile were added to each well immediately before measurements were taken (5 s per well).

Similar results were obtained when the two colorimetric tests were performed with four C. albicans strains. Figure 1 shows that the inhibition curves were both easily interpretable and correlated with the MIC₉₀s for the different strains determined by the standard broth macrodilution assay (Fig. 1A and B; Table 1).

Unexpectedly, divergent results were obtained for nine strains of A. fumigatus. The MTT test continued to generate interpretable inhibition curves, and MIC₉₀s ranged from 0.32 to 1.25 μg/ml (Fig. 2A). A standardized susceptibility test for A. fumigatus is not available, so established data are lacking for comparison. However, our MICs were compatible with those reported for Aspergillus strains in other studies (2, 3). In contrast, the Alamar Blue test did not generate interpretable inhibition curves for any of the Aspergillus strains. Six strains including the isolate from the American Type Culture Collection produced no significant fluorescent signal even in the absence of amphotericin B. Control experiments in which the metabolic activities of cells preincubated with Alamar Blue were subsequently assessed by the MTT test excluded any cytotoxic effect of Alamar Blue on the fungi. Two Aspergillus strains produced a weak Alamar Blue signal, but only one strain generated an acceptable starting value. For the last three strains, inhibition curves showed strong trailing, and 90% in-

### TABLE 1. MICs of amphotericin B for C. albicans strains determined by menadione-coupled MTT test, Alamar Blue assay, and macrodilution assay

<table>
<thead>
<tr>
<th>Strain</th>
<th>Alamar Blue assay</th>
<th>MTT test</th>
<th>Macrodilution assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>0815</td>
<td>0.32</td>
<td>0.32</td>
<td>0.156</td>
</tr>
<tr>
<td>R64</td>
<td>2.5</td>
<td>1.25–2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>3059</td>
<td>0.16</td>
<td>0.16–0.32</td>
<td>0.625</td>
</tr>
<tr>
<td>8116</td>
<td>0.32</td>
<td>0.16</td>
<td>0.32</td>
</tr>
</tbody>
</table>

*Three replicates of each assay were performed.*
When surface oxidase activity was assessed, high levels of activity were detected for all four C. albicans strains but not the three Aspergillus strains tested (Fig. 3). The absence of significant surface oxidase activity is thus one possible explanation for the poor reactivities of the A. fumigatus strains in the Alamar Blue test. Other reasons underlying the interstrain variations in the Alamar Blue test are unknown. Microscopically, no differences in germination and hyphal growth were discerned. Because the reactivities in the MTT test were similar (Fig. 2), the metabolic activities of the Aspergillus strains also appeared comparable to one another.

Because of these findings, our laboratory now routinely uses the MTT test for antifungal susceptibility testing. The test can also be performed in the presence of serum. The results obtained in a clinical setting have thus far been highly promising. Work is under way to test how the assay performs with other fungal pathogens.

REFERENCES


