RAPID QUANTITATIVE METHOD FOR MEASURING THE SENSITIVITY OF
BLASTOMYCES DERMAITIDIS TO FUNGISTATIC AGENTS

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ABSTRACT

GUIDRY, D. J. (Louisiana State University School of Medicine, New Orleans) and FINDLAY MAIER. Rapid quantitative method for measuring the sensitivity of Blastomyces dermatitidis to fungistatic agents. J. Bacteriol. 85:504–508. 1963.—Isolates of Blastomyces dermatitidis (yeast phase) were grown in continuous shake liquid culture. When sufficient cells were added to impart an initial optical density of 0.100 to the inoculated medium, growth was rapid, and the time required to assay fungistatic agents was reduced to 48 hr. Standard curves on light transmittance and direct cell count facilitated preparation of inoculum and measurement of growth. Generation time was determined for various growth phases of B. dermatitidis, and the data were applied to selection of the optimal period of growth for assaying fungistatic agents. Four isolates of B. dermatitidis were tested for their sensitivity to amphotericin and 2-hydroxystilbamidine. An amphotericin B concentration of 1.4 μg/ml was sufficient for maximal inhibition of all isolates tested; there were differences among isolates in sensitivity to lower concentrations of the drug. A 2-hydroxystilbamidine concentration of 62.5 μg/ml was sufficient for maximal inhibition of two isolates; the two remaining isolates required a concentration of 125 μg/ml. A latent period in inhibition of growth occurred when amphotericin B and 2-hydroxystilbamidine were added to cultures of B. dermatitidis. This latent period, together with generation time of the yeast cells, determined the maximal degree of inhibition which could be attained.

Preliminary experiments with Blastomyces dermatitidis in continuous shake liquid culture showed that growth could be detected after 18 hr of incubation. It was apparent that this method might prove feasible for measuring sensitivity to fungistatic agents and substantially reduce the time required for assay.

A number of investigators have used liquid shake cultures to study yeast growth. Atkin, Schults, and Frey (1942) prepared calibration curves for standardizing yeast inoculum and measuring growth on the basis of optical density (OD). Halliday and McCoy (1955) used liquid shake cultures to study the biotin requirements of B. dermatitidis; Pine (1957) studied growth of Histoplasma capsulatum; and Ahearn et al. (1960) performed assimilation tests on several species of yeasts.

The workers cited above employed culture tubes and colorimeter tubes of various sizes. An experience with the dermatophytes reported by Guidry and Trelles (1962) demonstrated that better aeration of cultures can be attained in Erlenmeyer flasks than in culture tubes. The method presented here used a standardized inoculum, which was placed in flasks of liquid medium and incubated in a shaker bath. Growth of B. dermatitidis was rapid; in vitro assay of fungistatic agents required only 48 hr; and useful data could be collected during assay.

MATERIALS AND METHODS

Isolates of B. dermatitidis were maintained in continuous shake liquid culture at 37 C. The yeast-phase cultures were grown in 125-ml Erlenmeyer flasks containing 10 ml of broth. Sabouraud’s dextrose broth (1% peptone and 3% dextrose) was employed in maintaining cultures and in growth experiments. Cultures were incubated in a reciprocating shaker bath (model RW 653; New Brunswick Scientific Co., New Brunswick, N.J.) operated at 72 horizontal strokes per min.

The inoculum for growth experiments was prepared from 3-day-old cultures obtained by
TABLE 1. Growth of Blastomyces dermatitidis in continuous shake liquid culture

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Optical density of cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>BdM</td>
<td>0.100 0.119 0.125 0.138 0.171 0.342 0.569 0.720</td>
</tr>
<tr>
<td>BdS</td>
<td>0.102 0.106 0.131 0.157 0.185 0.414 0.588 1.000</td>
</tr>
<tr>
<td>BdT</td>
<td>0.102 0.115 0.123 0.141 0.161 0.444 0.917 1.022</td>
</tr>
<tr>
<td>BdW</td>
<td>0.103 0.069 0.086 0.147 0.162 0.604 0.917 1.022</td>
</tr>
</tbody>
</table>

FIG. 1. Growth of BdW isolate of Blastomyces dermatitidis in continuous shake liquid culture.

the above method. Growth from several flasks was pooled, washed three times by centrifugation with 5% dextrose solution, and resuspended in Sabouraud's dextrose broth to a final OD of 0.100.

Growth of *B. dermatitidis* was measured by determining the OD of samples removed from each flask at selected intervals of time. The samples were washed once, resuspended in 5% dextrose solution, and OD was measured at a wavelength of 500 nm on a Coleman Junior spectrophotometer (model 6A).

Commercially available preparations of amphotericin B and 2-hydroxystilbamidine were used. Weighed samples of the drugs were transferred to 15-ml screw-cap tubes and refrigerated until used. When needed, a 5% dextrose solution was employed to prepare stock concentrations of each drug equal to ten times the desired flask concentration. For in vitro assay, 1.0 ml of each stock concentration was added to 24-hr flask cultures of *B. dermatitidis*, which contained 9.0 ml of Sabouraud's dextrose broth. Distilled water (0.34 ml) was also added to compensate for evaporation during the 24-hr incubation period.

RESULTS

An initial inoculum of *B. dermatitidis*, imparting an OD of 0.100 (2 × 10⁴ cells per ml) to the culture medium, was found to be the most satisfactory. Significant changes in the OD of the cultures occurred after 18 to 24 hr of incubation.

Table 1 shows the growth of four isolates of *B. dermatitidis* over a 96-hr period. Isolates were obtained from active cases of blastomycosis and have been maintained in the laboratory for 3 to 5 years. Each isolate is designated by initials representing genus, species, and the patient's name. Adjustment of the initial inoculum was facilitated by the use of a standard light-transmittance curve. Differences in the rate of growth of the isolates were not apparent until after 48 hr of incubation. Cultures showed a two- to fourfold increase in OD during the incubation period (24 to 48 hr). The growth rates decreased sharply after 48 hr, when cultures appeared to be approaching a stationary phase.

Changes in rate of growth are shown graphically in Fig. 1, where growth of the BdW isolate has been plotted. The lag in growth during the first 24 hr, as well as the decrease in rate of growth during the 48- to 96-hr period, is readily apparent. Thus, the *B. dermatitidis* yeast shows a lag phase, a phase of accelerated growth, and a negative growth acceleration phase.

Generation time was calculated for the isolates shown in Table 1. During 24 to 48 hr of incubation, values ranged from 11 hr for the BdW isolate to 24 hr for the BdM isolate. Generation time increased to about 29 hr during 48 to 72 hr of incubation, and to 45 hr during 72 to 96 hr of incubation.

The fungistatic activity of amphotericin B and 2-hydroxystilbamidine on *B. dermatitidis* was measured during the period of most active growth. Freshly inoculated cultures were incubated for 24 hr; various concentrations of
Drug were added, and the fungistatic effect measured 24 hr later after 48 hr of incubation.

Figure 2 shows the fungistatic effect of amphotericin B on the BdM isolate of B. dermatitidis. The pH of the culture medium in both control and test flasks remained essentially unchanged. None of the isolates produced a significant change in the pH of the culture medium.

The fungistatic effect at each drug concentration was determined by comparing growth in control flasks during the 24- and 48-hr incubation periods with growth in test flasks. The values obtained, expressed as per cent inhibition of growth, are presented in Fig. 3 for various concentrations of amphotericin B. Growth in the 24-hr control flasks was about the same for the three isolates shown in Fig. 3. Growth of the 48-hr controls was greater for the BdM and BdS isolates (average OD was 0.603) than for the BdT isolate (OD was 0.509). This difference in growth rate appears to have little influence on sensitivity of the isolates to amphotericin B, since the BdM isolate is uniformly more sensitive than the BdT isolate and the BdS isolate appears less sensitive at lower concentrations. If a real difference in sensitivity to amphotericin B exists among isolates, it is manifested only at low concentrations of the drug where the fungistatic effect is approximately 50% or less than 50%. Converging of the lines at higher concentrations of amphotericin B and failure to obtain 100% inhibition are characteristics inherent in the method of testing and will be discussed in relation to results obtained with 2-hydroxystilbamidine.

The fungistatic effect of 2-hydroxystilbamidine was studied in the same manner as amphotericin B. BdW was the most sensitive of the four isolates tested (Fig. 4). Control growth was considerably less for BdW than for the other isolates. However, differences in sensitivity cannot be explained entirely on the basis of the number of yeast cells, for the BdT isolate grew as well as the BdM isolate, and the difference in their sensitivity to a drug concentration of 62.5 μg/ml is obvious. Generation time may be of greater importance than total cell count in influencing the sensitivity of B. dermatitidis to a drug. In Fig. 4, the BdM and BdS isolates had a generation time of 11 hr, and the BdT and BdW isolates had generation times of 22 and 24 hr, respectively.

Failure to obtain 100% inhibition of growth (Fig. 3 and 4) is due to cell multiplication during the time required for drug action to become
TABLE 2. Cell multiplication during the 2-hr latent period required for 2-hydroxystilbamidine to penetrate the cell wall

<table>
<thead>
<tr>
<th>Isolate</th>
<th>OD of control flask</th>
<th>Generation time at 24 hr</th>
<th>Growth expected at 26 hr (OD)</th>
<th>Actual growth at 26 hr (OD)</th>
<th>Maximal inhibition possible %</th>
</tr>
</thead>
<tbody>
<tr>
<td>BdM</td>
<td>0.168</td>
<td>0.678</td>
<td>11</td>
<td>0.196</td>
<td>0.222*</td>
</tr>
<tr>
<td>BdT</td>
<td>0.310</td>
<td>0.620</td>
<td>22</td>
<td>0.361</td>
<td>0.337†</td>
</tr>
<tr>
<td>BdS</td>
<td>0.237</td>
<td>0.824</td>
<td>11</td>
<td>0.317</td>
<td>0.319*</td>
</tr>
<tr>
<td>BdW</td>
<td>0.134</td>
<td>0.229</td>
<td>24</td>
<td>0.142</td>
<td>0.140†</td>
</tr>
</tbody>
</table>

* Growth occurring at drug concentration of 125.0 μg/ml.
† Calculated as follows: 100% — [(growth expected at 26 hr — growth in 24 hr)/(growth in 48 hr — growth in 24 hr)] x 100.
† Growth occurring at drug concentration of 62.5 μg/ml.

effective. Pope and Christison (1953) reported that oxygen consumption by B. dermatitidis was not affected by stilbamidine until 2 hr after addition of the drug to a cell suspension; they attributed the latent period to slow penetration of the drug into the cells. In Table 2, it is assumed that a 2-hr latent period occurred between addition of the drug and complete fungistasis. Generation time was calculated from the number of cells equivalent to OD values in the control flasks at 24 and 48 hr. A comparison between growth expected at 26 hr with growth in flasks containing sufficient drug to exert complete fungistasis shows that growth of the four isolates was completely inhibited approximately 2 hr after addition of the drug. The calculated values for maximal inhibition (Table 2) and the experimental values (Fig. 4) are in close agreement.

DISCUSSION

Continuous shake liquid cultures provide a simple and rapid means for measuring the effect of fungistatic agents on growth of B. dermatitidis. The agents are subjected to unfavorable factors, such as pH and temperature, for a minimal period of time. Growth rate and total cell count can be determined at any time during assay, providing information concerning their influence on sensitivity of Blastomyces isolates to the drug under evaluation. Differences among isolates in sensitivity to fungistatic agents can be detected which would not be apparent in tests performed on semisolid medium.

Differences in testing methods preclude a valid comparison between the effective drug concentrations presented here and data appearing in the literature. Drouhet and Wilkinson (1957) used blood-eysteine-heart infusion agar to measure the fungistatic effect of amphotericin B on four isolates of B. dermatitidis. Three isolates were inhibited by an amphotericin B concentration of 0.7 μg/ml, and one isolate was inhibited by 1.5 μg/ml. Halde et al. (1957), using an asparagine synthetic broth, found that an amphotericin B concentration of 0.5 μg/ml completely inhibited growth of B. dermatitidis. Seabury and Dascomb (1958) used Sabouraud’s dextrose agar and found that 0.06 μg/ml of amphotericin B was adequate for inhibiting growth.

McMillen, Kushner, and Snapper (1955) used Sabouraud’s dextrose agar slants to study the fungistatic effect of six diamidine compounds on B. dermatitidis. Of the 12 isolates tested, 8 were completely inhibited by a 2-hydroxystilbamidine concentration of 5.0 μg/ml, two isolates required 25.0 μg/ml for inhibition, and two isolated required 50 μg/ml. The reports cited involved assay periods of 5 to 10 days.

The latent period reported for stilbamidine inhibition of O2 consumption by Blastomyces cells (Pope and Christison, 1953) agrees with the latent period in fungistasis reported here. A similar latent period in the experiments with amphotericin B indicates that slow penetration into the Blastomyces cells is not limited to diamidine compounds.

Pope and Christison (1953) found that with a glucose substrate a stilbamidine concentration of 200.0 μg/ml depressed O2 consumption 95%. Although the concentration of Blastomyces cells in the Warburg vessels was greater than in the Erlenmeyer flasks, the relationship between the amount of stilbamidine required to inhibit O2 consumption and the amount required to inhibit growth is obvious. Combining growth studies in liquid shake culture with studies on oxidative metabolism should prove valuable in evaluating the effect and elucidating the mode of action of both stimulatory and inhibitory compounds. The method presented here, along with a previous report on preparation of homogeneous mycelial suspensions (Guidry and
Trelles, 1962), will facilitate study of the dimorphic pathogenic fungi.

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LITERATURE CITED


