Inhibition of Fungal Growth in the Cultural Isolation of Mycobacteria

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Received for publication 1 May 1972

Antifungal antibiotics were compared to determine their usefulness in primary mycobacterial cultures. Amphotericin B was found to be more effective in preventing fungal growth from bovine fecal specimens than were cycloheximide, nystatin, and tetracycline. Amphotericin B did not affect the growth rate of the following Mycobacterium species: M. avium, M. bovis, M. intracellulare, M. paratuberculosis, M. phlei, or M. tuberculosis, but it inhibited the growth of M. fortuitum. There was no observable effect on numbers of colonies of M. paratuberculosis on primary isolation from fecal specimens. It is recommended that, for the primary isolation of pathogenic mycobacteria from specimens likely to contain fungi, the inoculum should be pretreated with benzalkonium chloride, followed by mixing with amphotericin B or inoculation onto media containing amphotericin B.

The use of benzalkonium chloride for decontamination of specimens to be cultivated for the isolation of pathogenic mycobacteria is an accepted technique (1–4, 7). Although this agent prevents the growth of most of the unwanted bacteria, it is ineffective against fungi. Fecal specimens to be cultured for the isolation of Mycobacterium paratuberculosis (2, 3) frequently contain fungi, especially mucor species, which cannot successfully be controlled by benzalkonium chloride. Furthermore, sputum specimens from some humans suspected of being tuberculous regularly contain fungi.

The prevalence of fungi in specimens being cultured to detect mycobacteria necessitated a study to find an agent with antifungal activity, but without antimycobacterial activity for use in the control of fungi in such specimens.

MATERIALS AND METHODS

Comparison of antifungal antibiotics. Each of two bovine fecal specimens containing fungi were suspended in 20 volumes of water and allowed to stand 2 hr. A 1-ml amount of supernatant fluid from each suspension was then added to 1.0 ml of each antibiotic dilution and to 1.0 ml of water. The antibiotic dilutions were: amphotericin B (Pungazine, E. R. Squibb & Sons, Inc., New York), 5,000, 500, and 50 μg/ml; cycloheximide (Actidione, Upjohn Co., Kalamazoo, Mich.), 10,000, 1,000, and 100 μg/ml; nystatin (Mycostatin, E. R. Squibb & Sons, Inc., New York), 17,000, 1,700, and 170 μg/ml; and tetracycline HCl (Achromycin, Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York), 50,000, 5,000, and 500 μg/ml. The suspensions were allowed to stand for 20 hr at 24 C, after which 30.0 ml of 0.3% benzalkonium chloride (Zephiran, Winthrop Laboratories, New York) solution was added to each and allowed to stand an additional 24 hr at 24 C to eliminate contaminating bacteria. The resulting sediments were pipetted, without removal of the decontaminants, in 0.2-ml portions, onto each of two slants of mycobactin-egg yolk (MEY) medium (2). All slants were incubated for 2 weeks at 38 C.

Effect of amphotericin B on various mycobacterial species. Tubes of serum agar (6) containing 2 μg of ferric mycobactin per ml and 1,000, 500, 100, 50, 10, 5.0, 1.0, or 0 μg of amphotericin B per ml were inoculated with suspensions of M. tuberculosis, M. paratuberculosis, M. bovis, M. avium, M. intracellularare, M. phlei, and M. fortuitum. All tubes were incubated at 38 C, and growth was observed visually and recorded at 4, 6, and 8 weeks.

Effect of amphotericin B on primary cultures from fecal specimens. Fecal specimens from 788 cattle were submitted for cultural examination for M. paratuberculosis. The cattle were located on 29 farms in 10 states and Puerto Rico. Approximately 1 g of each fecal specimen was suspended in 30 to 40 volumes of distilled water and then allowed to stand 1 hr for sedimentation of the large particles. A 5.0-ml amount of the supernatant suspension was added to 35.0 ml of 0.3% benzalkonium chloride solution. After 16 to 20 hr at 24 C, the sediment which formed without centrifugation was distributed onto two slants each of MEY medium containing 50, 1.0, and
0 µg/ml of amphotericin B. All slants were incubated at 38 C for 12 weeks or discarded when overgrown with fungi.

**Addition of amphotericin B to inoculum.** Duplicate portions, A and B, were used of specimens from one group of 250 cattle whose fecal specimens persistently produced fungal growth on medium slants. Each portion was treated with 0.3% benzalkonium chloride as above. Each of four slants of MEY medium was inoculated with 0.2 ml of the sediment which formed in portions A after benzalkonium chloride treatment. Portion B from each specimen was centrifuged at 2130 RCF for 30 min after benzalkonium chloride treatment and then resuspended in 1.0 ml of sterile water containing 1,000 µg of amphotericin B. Each of four slants of MEY medium was inoculated with 0.2 ml of amphotericin B-treated portion B. All slants were incubated at 38 C for 12 weeks or discarded when covered by fungi.

**RESULTS**

**Comparison of antifungal antibiotics.** Of the four antibiotics used, only amphotericin B prevented fungal contamination at all dilutions. Some fungal growth occurred after cycloheximide treatment at all concentrations, after nystatin treatment at 170 and 1,700 µg/ml but not after 17,000 µg/ml, and after tetracycline treatment at 400 µg/ml, but not at higher concentrations. Although the last three antibiotics did not prevent all fungal growth at their lower concentrations, they did reduce the amount of fungal growth compared to the water-treated controls.

**Effect of amphotericin B on mycobacterial species.** No difference was detected in the rate or total amount of bacterial growth of any species of mycobacteria by any level of amphotericin B tested, except that *M. fortuitum* was slightly inhibited at the 500 µg/ml level and almost totally inhibited at the 1,000 µg/ml level.

**Effect of amphotericin B on primary cultures from fecal specimens.** Colonies of *M. paratuberculosis* grew on one or more tubes from 43 of the 788 fecal specimens cultured. Table 1 shows the numbers of slants with fungal growth at each amphotericin B level from the 745 specimens without *M. paratuberculosis*. It should be noted that, although both tubes contain fungal growth in 11 instances at the 50 µg/ml level, the fungal colonies remained small after 12 weeks of incubation and probably would not have prevented the observation of mycobacterial colonies if they had been present. The effect of amphotericin B on numbers of colonies of *M. paratuberculosis* from 27 of 43 fecal specimens is presented in Table 2. Specimens from which the numbers of colonies were too numerous to count or where one or more slants were contaminated were not included. The analysis of variance of the square root of the colony counts provides no evidence of a significant effect on primary colony count when amphotericin B is incorporated in the primary culture medium, even at the 50 µg/ml level. The nonsignificant interaction, coupled with a significant specimen effect, indicates a consistent behavior from one level of amphotericin B to another, even though there is a significant specimen-to-specimen variability.

**Addition of amphotericin B to inoculum.** One or more of the slants from 245 of the 250 specimens were covered by fungi when amphotericin B was not added to the inoculum, but slants from only nine specimens contained fungal growth when the inoculum was suspended in amphotericin B before inoculation. *M. paratuberculosis* was isolated from two of the specimens treated with amphotericin B, but no isolation was made from the same specimens without the treatment.

**DISCUSSION**

From the above observations, it is apparent that amphotericin B is effective for the prevention of fungal contamination on media used to culture mycobacteria. Moreover, there was no observable effect on numbers of colonies of *M. paratuberculosis* on primary isolation.

**Table 1. Occurrence of fungal growth on cultures from 745 bovine fecal specimens on media with different amphotericin B contents**

<table>
<thead>
<tr>
<th>Fungi present</th>
<th>Amphotericin B in medium (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Neither tube</td>
<td>617</td>
</tr>
<tr>
<td>One tube</td>
<td>85</td>
</tr>
<tr>
<td>Both tubes</td>
<td>43</td>
</tr>
</tbody>
</table>

**Table 2. Analysis of variance of the square root of colony counts of Mycobacterium paratuberculosis on primary cultures from fecal specimens**

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees of freedom</th>
<th>Mean square*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B concn</td>
<td>2</td>
<td>1.55 NS</td>
</tr>
<tr>
<td>Specimens</td>
<td>26</td>
<td>46.33**</td>
</tr>
<tr>
<td>Amphotericin B x specimens</td>
<td>52</td>
<td>1.82 NS</td>
</tr>
<tr>
<td>Error</td>
<td>81</td>
<td>1.54</td>
</tr>
</tbody>
</table>

*NS, No significant difference among specimens (P > 0.10); **, significant difference among specimens (P < 0.01).
and no change in the amount of growth of other mycobacterial species except *M. fortuitum*. The latter effect is not significant when amphotericin B is to be used in conjunction with benzalkonium chloride, because *M. fortuitum* and other Runyon group IV organisms seldom survive the benzalkonium chloride treatment (4). Therefore, for likelihood of pathogenic mycobacteria in inoculum be B or inoculation followed by mixing with amphotericin B or inoculation onto medium containing amphotericin B.

A study of the stability of amphotericin B incorporated in complex media (5) failed to detect a loss of antifungal activity when the media were stored at 4 C for 1 week, but 33 to 42% of the activity was lost after 1 week at 37 C. Since most cultures of fungi are completely inhibited by 1 μg of amphotericin B per ml, there would still be sufficient antifungal activity left after 2 months of incubation of media initially containing 50 μg of amphotericin B per ml. Since there is some loss in antifungal activity of amphotericin B stored in solution, the incorporation of this antibiotic in the media for mycobacterial isolation probably should be limited to those laboratories where mycobacterial medium is prepared frequently and stored for only short periods. When small numbers of specimens are being cultured, and medium is stored for long periods, a sterile solution of amphotericin B can be kept frozen and be added to the inoculum immediately before inoculation.

ACKNOWLEDGMENTS

We thank Gordon D. Booth for the statistical analysis, and Richard D. Ness, Florence M. Gjertson, and Kathryn B. Meredith for their technical assistance.

LITERATURE CITED