Rapid Radiometric Method for Determining Drug Susceptibility of Mycobacterium avium-intracellulare

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A rapid radiometric method for susceptibility testing of Mycobacterium avium-intracellulare to eight chemotherapeutic agents was compared with a conventional method. Results were available within 72 h by radiometric testing in contrast to 21 days by the conventional method. The radiometric and conventional methods agreed in 61% of the tests, but growth inhibition of ≥50% was detectable only by radiometric testing in an additional 36.5% of the tests. In only 2.5% of the tests was the radiometric method unable to detect complete inhibition shown by the conventional method. Quantifiable increases in inhibition with increasing concentration of isoniazid were more frequently detectable by the radiometric method than by conventional testing. The radiometric method is a simple, rapid, and quantitative test for drug susceptibility of mycobacteria and warrants further investigation.

Mycobacterium avium-intracellulare is an important cause of human mycobacterial infection in the United States (6, 22). Furthermore, M. avium-intracellulare infections are recognized with increasing frequency in immunocompromised patients (16). Chemotherapy with up to six antimycobacterial drugs simultaneously has frequently proven to be unsuccessful, and surgical therapy is beneficial in only a minority of selected patients (2, 7, 14, 17, 23). Establishing effective chemotherapy for this infection has been hampered in part by the prolonged time required to determine in vitro susceptibility.

In 1974, Buddemeyer described a radiometric method for monitoring bacterial growth that is remarkably simple and inexpensive and utilizes materials widely available in most hospital laboratories (3). In this paper, we report the adaptation of the Buddemeyer technique to the measurement of susceptibility of M. avium-intracellulare to eight chemotherapeutic agents, and we compare this test with the conventional agar disk diffusion test.

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MATERIALS AND METHODS

Organisms. Nineteen clinical isolates of M. avium-intracellulare were obtained from the Tuberculosis Research and Reference Laboratory, Tampa Branch Laboratory, Tampa, Fla., and one isolate (ATCC 23068) was purchased from the American Type Culture Collection, Rockville, Md.

Media. The isolates were inoculated in 7H9 Middlebrook liquid medium supplemented with ADC (albumin-dextrose-catalase) or OADC (oleic acid-bovine albumin-dextrose-catalase) (BBL Microbiology Systems, Cockeysville, Md.) in plastic tubes (17 by 100 mm) and incubated at 37°C with 5 to 10% CO2 for at least 4 to 5 days. Turbidity was adjusted to the density of McFarland no. 1 (1), approximately 106 colony-forming units per ml. Proskauer Beck medium (Difco Laboratories, Detroit, Mich.) with or without glycerol was also used during preliminary studies.

Antimycobacterial agents. The stock solutions and various drug concentrations selected were prepared by the Centers for Disease Control procedure (20). The antimycobacterial agents were provided by the following companies: isoniazid (INH), streptomycin sulfate (SM), cycloserine (CS), and paraaminosalicylic acid (PAS) were from Eli Lilly & Co., Indianapolis, Ind.; ethambutol (EMB) was from Lederle Laboratories, Pearl River, N.Y.; rifampin (RF) was from Dow Pharmaceuticals, Indianapolis, Ind.; ethionamide (ETA) was from Ives Laboratories Inc., New York, N.Y.; and clofazimine (CFZ) was from Ciba Pharmaceutical Co., Summit, N.J.

Sensi-Disc microbial susceptibility disks (BBL) were utilized for the conventional method, except for the following disks, which were prepared in our labo-
ratory: INH, 5 μg/ml; CS, 20 μg/ml; and CFZ, 1 μg/ml. Because CFZ diffused poorly from the disks, an agar dilution method was performed for this drug.

**14C-labeled substrates.** The following 14C-labeled substrates were purchased from Amersham Corp., Arlington, Ill., and evaluated for counting efficiency: sodium salts of [U-14C]acetate, [14C]formic acid, and [U-14C]pyruvic acid and D-[U-14C]glucose, [U-14C]glycerol, and [U-14C]glucose.

**Radiometric method.** Buddemeyer’s radiometric method has been described in detail previously (3–5). Briefly, the components consisted of an inner sterile glass vial surrounded by a filter paper saturated with concentrated toluene solution and moistened with 2 M NaOH. The glass vial-filter paper component was placed in an outer polyethylene vial.

On the day of the experiment, previously prepared broth cultures of **M. avium-intracellulare** and 1 μCi of 14C-labeled substrate were placed in the inner vial. 14CO2 evolution was measured with an automatic liquid scintillation spectrometer model 3380 (Packard Instrument Co., Inc., Downers Grove, Ill.) modified to operate at 37°C. All experiments were done in triplicate, and samples were counted every 1 to 2 h for 72 to 96 h.

**Antimicrobial agent-impregnated disk method.** The conventional agar disk diffusion test was performed as described by Wayne and Krasnow (21). Samples of 100 μl of 10−4 and 10−3 colony-forming units per ml of **M. avium-intracellulare** were inoculated into quadrants of plates of Middlebrook 7H10 agar with OADC enrichment. The plates were incubated at 37°C with 5% to 10% CO2, and the results were read at 3, 4, and 6 weeks.

**Calculation of replication time and percent inhibition.** Buddemeyer’s formulas (4) for calculation of replication time (Tc) and percent inhibition were utilized as follows: Tc = (t2 - t1)/logC2 - logC1 for the interval t1 to t2 with the best log-linear fit over two generations, where t1 and t2 indicate the time interval (in minutes), and C1 and C2 are the counts per minute at each point. The replication time is inferred from the doubling time of 14CO2 production. Percent inhibition (PI) was calculated as PI = 100(1 - Tc/TA) for the inter maximum inhibition over two generations of control growth, where Tc and TA are replication times of control and treated samples, respectively.

The data were collected, displayed, and analyzed with computer assistance (B. G. Yangco, J. A. Madden, E. A. Eitkman, D. A. Solomon, and S. C. Deresinski, J. Nucl. Med. 21:p72, 1980).

**Criteria utilized for evaluation of conventional and radiometric methods.** (i) Conventional method. It has been generally accepted that when 1% or more of the organisms which grow on the control are observed to grow in presence of a drug, for clinical purposes, that particular drug is not, or soon may not be, valuable for treatment (20). Based upon this premise and for the purpose of comparing this method with the radiometric test, we have grouped the Centers for Disease Control criteria (20) into two groups. In the first group, there was no growth to ≤5 colonies, corresponding to <1% of control growth in the medium containing the drug. In the second group, there was growth of >5 colonies to ≥1%, corresponding to ≥1% of control growth in the medium containing the drug. (ii) Radiometric method. A previous report with other human pathogens suggested that inhibition of at least 50% of the metabolic activity of the organisms was indicative of susceptibility to the drug by the radiometric method (9). Thus, two preliminary categories were formed for comparison with the conventional method: <50% and ≥50% inhibition.

**RESULTS**

**Radiometric method: preliminary studies.** To determine the appropriate method, preliminary studies were done in which the following optimal conditions were determined. A 4- to 7-day-old inoculum diluted to yield 106 or 107 colony-forming units per ml in a ratio of 1:5 (broth culture/liquid medium) produced optimal control growth (Fig 1A). [14C]Formate consistently produced satisfactory control, 14CO2 evolution in a random sample of isolates (Fig. 1B). Middlebrook 7H9 plus ADC or OADC medium was superior to Proskauer Beck medium with or without glycerol (Fig. 1C).

**Comparison between the conventional and radiometric methods.** Of the 20 isolates, 2 were excluded in the final analysis because of poor growth in the control group.

Figure 2 shows typical examples of experimental results illustrating no inhibition or inhibition of growth of **M. avium-intracellulare** in response to antimycobacterial agents.

Table 1 shows a comparison between the radiometric and conventional methods. Overall, there was 61% agreement for all of the drugs between the two tests. There was a good agreement of results for isolates tested with INH at 0.2 μg/ml (100%), PAS (94%), RF (83%), SM at 10 μg/ml (78%), ETA (61%), and CFZ (59%). In an additional 36.5% of the tests, growth inhibition of ≥50% was detectable only by the radiometric method. This was most notable with CS (83% of isolates), but was also evident in isolates treated with INH at 5 μg/ml (61%), SM at 2 μg/ml and EMB (both 56%), and INH at 1 μg/ml (50%).

In only 2.5% (5 of 197) of the tests where there was complete inhibition (<1% growth compared with controls) by the conventional method was there no significant inhibition (<50% inhibition) by the radiometric method (Table 1).

An increase in percent inhibition detectable by the radiometric method was noted in 94% of isolates treated with increasing concentrations of INH (Table 1). With the conventional method, this drug effect was observed only in half of the isolates (Table 1).

**Susceptibility of M. avium-intracellulare**
as determined by the radiometric method. Figure 3 shows the drug susceptibility pattern of M. avium-intracellulare as determined by the radiometric method. Based on these criteria, INH at 5 μg/ml, RF, SM at 2 μg/ml, SM at 10 μg/ml, EMB, CS, and CFZ appeared to be active in vitro against the M. avium-intracellulare isolates tested.
Replication time of M. avium-intracellulare. Replication time of M. avium-intracellulare as determined by Buddemeyer's method was 13.7 ± 3.5 h. This resembles the generation times reported for slow-growing mycobacteria such as Mycobacterium tuberculosis (15 h) and Mycobacterium kansasii (13 h) on 7H9 medium (8).

DISCUSSION

Detection of drug resistance and screening for more effective chemotherapeutic agents are important indications for drug susceptibility testing of mycobacteria (8). However, laboratory standardization of the current method has been difficult (20), and translation of laboratory results into clinical application is uncertain. Furthermore, the prolonged time required for the conventional method, added to the initial time needed to isolate the organism from clinical material, contributes to delay in instituting appropriate chemotherapy. For these reasons, there is a need for a method that is simple, rapid, quantitative, and clinically relevant.

The radiometric method that we report reduced the time for drug susceptibility measurement by 85%. The method was at least as sensitive as the conventional method in detecting growth inhibition. Agreement between the two methods was highest with INH at 0.2 μg/ml, SM at 10 μg/ml, RF, PAS, ETA, and CFZ. Discrepancies between results of the two methods were noted with CS, EMB, INH at 1 and 5 μg/ml, and

Fig. 2. Examples of radiometric measurement of metabolic activity of M. avium-intracellulare strain "Bailey." Percent inhibition for INH at 0.2 μg/ml and RF at 1 μg/ml were 11 and 96%, respectively.

Table 1. Comparison between the radiometric and conventional methods of drug susceptibility testing of 18 clinical isolates of M. avium-intracellulare

<table>
<thead>
<tr>
<th>Drug (μg/ml)</th>
<th>Susceptible by both methods</th>
<th>Resistant by both methods</th>
<th>Radiometric method only</th>
<th>Conventional method only</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH (0.2)</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>0</td>
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<tr>
<td>INH (1)</td>
<td>0</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>INH (5)</td>
<td>5</td>
<td>2</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>RF (1)</td>
<td>15</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>SM (2)</td>
<td>8</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>SM (10)</td>
<td>14</td>
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<td>4</td>
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</tr>
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<td>EMB (6)</td>
<td>3</td>
<td>5</td>
<td>10</td>
<td>0</td>
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<td>PAS (2)</td>
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<td>0</td>
<td>1</td>
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<td>CS (20)</td>
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<td>15</td>
<td>0</td>
</tr>
<tr>
<td>CFZ (1)</td>
<td>10</td>
<td>0</td>
<td>5</td>
<td>2</td>
</tr>
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</table>

*Results indicate the number of isolates. The terms susceptible and resistant are used as defined in the text.

Fig. 3. Drug susceptibility pattern of M. avium-intracellulare by the radiometric method. Each bar with a vertical line inside represents the mean and standard deviation of the percent inhibition of 18 clinical isolates.
SM at 2 μg/ml. CS showed the greatest discrepancy among the drugs tested. By the radiometric method, significant inhibition was observed in 83% of the CS-treated isolates, whereas by conventional testing only 17% of the isolates showed significant growth inhibition. Explanation for the differences is not known, but may be due to: (i) degradation of antimycobacterial drugs in the culture medium over a period of time during conventional testing (18); (ii) emergence of resistant mutants; (iii) presence of mycobacterial substances such as mycobactin, which reverses the inhibitory effect of CS (19); (iv) poor diffusibility of drugs from the disk to the agar medium as observed with CFZ (Yangco, unpublished data); or (v) technical variation. Irregular in vitro results (11) and lack of correlation between conventional and radiometric methods (15) with EMB have been observed. These discrepancies may be due to reduced cell cohesion in the presence of EMB leading to an increase in colony-forming units early in the incubation period (13) and also may be due to the effect of culture media on bacterial susceptibility to EMB (10). On the other hand, in few cases did the conventional test demonstrate inhibition not detected radiometrically. Thus, technical factors did not significantly interfere with radiometric testing.

The radiometric method can also measure graded change in growth rate in response to various drug concentrations. The clinical significance of this attribute will require further investigation. A further area for clinical research is the definition of optimal criteria of clinical importance for susceptibility both by radiometric and conventional tests. No appropriate animal model or human studies for evaluating the effects of antmycobacterial agents on M. avium-intracellulare have been reported. In future in vivo (animal or human) drug trials, we hope to study the correlation of outcome with results of both the conventional and the radiometric method of susceptibility testing. An alternative approach to radiometric drug susceptibility determination (12; R. H. Lusk, L. F. Laskowski, and J. J. Marr, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 20th, New Orleans, La., abstr. no. 397, 1980) will require similar evaluation. If the radiometric technique proves predictive of therapeutic success, its speed, simplicity, and quantitative results could make it the method of choice.

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LITERATURE CITED