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Rapid Radiometric Method of Testing Susceptibility of Mycobacteria and Slow-Growing Fungi to Antimicrobial Agents

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[^{3}H]uracil incorporation into the ribonucleic acid (RNA) of mycobacteria and [^{3}H]guanine incorporation into the RNA of fungi were used as measures of cell viability in the presence of several antimicrobial agents. There appeared to be an excellent correlation between traditional susceptibility tests, which required 1 to 4 weeks, and tests of the inhibition of incorporation of RNA precursors, which were completed in 4 to 48 h. The radiometric method may be useful in rapidly determining the susceptibility of slow-growing organisms to antimicrobial agents.

Conventional antibiotic susceptibility testing of microorganisms use agar diffusion, agar dilution, or broth dilution methods (1). All of these techniques depend on visible growth of the organisms and therefore require prolonged incubation, particularly when they are done on slow-growing organisms such as mycobacteria or certain fungi.

Recently DeLand and others have developed a radiometric method to detect bacterial growth and antibiotic susceptibility by measuring the release of radiolabeled CO_2 from bacterial cultures which have metabolized uniformly labeled [^{14}C]glucose (3, 4). In those studies, the inhibition of the bacterial metabolism of radioactive glucose was directly correlated with susceptibility of the organisms to a variety of antibiotics.

It seemed to us that radiometric determination of antibiotic susceptibility might be particularly useful in slow-growing organisms such as mycobacteria and fungi. Our previous studies on fungi, which showed good correlation between broth dilution susceptiblity testing and the effects of antibiotics on macromolecular synthesis as determined by incorporation of radioactive amino acids into protein or nucleic acids into ribonucleic acid (RNA), suggested that this technique could be exploited for rapid susceptibility testing of other slow-growing organisms (5).

MATERIALS AND METHODS

Radiochemicals. Initially [^{14}C]glucose, [^{3}H]uracil [^{3}H]leucine, [^{3}H]uridine, and [^{3}H]glucose were evaluated in our test system. Based on the amount of radioactive material incorporated into the cells, reproducibility, and cost, [^{3}H]uracil (specific activity 18 Ci/mmol) was used in the experiments with mycobacteria, and [^{3}H]guanine (13 Ci/mmol) was used in the yeast studies. Isonicotinic acid hydrazide (INH) was purchased from Sigma Chemical Co., St. Louis, Mo. Streptomycin was purchased from Charles Pfizer and Co., Inc., New York, N.Y. Ethambutol hydrochloride was kindly provided by J.M. Smith, Jr. (Lederle Laboratories, Pearl River, N.Y.). Rifampin (Rifadin) was obtained from Dow Chemical, Zionsville, Ind. Amphotericin B (AmB) as Fungizone was purchased from E.R. Squibb and Sons, Inc., Princeton, N.J.

Organisms and growth conditions. *Mycobacterium bovis* (BCG) and *M. bovis* (BCG-R), an INH-resistant mutant, were both obtained from Kenneth McClatchy, National Jewish Hospital, Denver, Colo. Clinical isolates of *M. kansasii* and *M. intracellulare* were obtained from the Center for Disease Control (CDC), Atlanta, Ga. All cultures were maintained on Lowenstein-Jensen medium at 37 C in 5% CO_2 and subcultured every 7 days. The strains of *Histoplasma capsulatum* (Downs) and *Blastomyces dermatitidis* were maintained in the yeast phase on 2% glucose, 10% yeast extract agar, and are part of the permanent fungus collection of this laboratory.

In the mycobacterial radiometric studies, several loopfuls of 7-day-old cultures were transferred from the Lowenstein-Jensen medium to distilled water, ground in a Teflon tissue grinder, and passed through a coarse scintered-glass filter (40- to 60-μm pore size). The optical density of the uniform suspension was adjusted to 0.1 at 460 nm on a Bausch & Lomb Spectronic 20; this corresponded to a colony count of 5×10^6 organisms per ml, and was our initial inoculum for the radioactive studies. The radioactive studies were done in standard 7H-11 liquid medium (6).

In the yeast experiments, 7-day-old yeast-phase cultures were transferred to Salvin broth (7) at a con-

centration of 10^5 cells per ml (hemocytometer counts) and then incubated at 37 C for 24 h before the experiments were performed.

Antimicrobial susceptibility tests. The susceptibility tests on the mycobacteria were performed in duplicate exactly as done in the diagnostic laboratory of Barnes Hospital, St. Louis, Mo., using the indirect agar procedure recommended by the CDC (2).

The yeast susceptibility tests were done in duplicate by a modification of the tube dilution method in Salvin broth (5). The fungi were incubated with different concentrations of AmB, and the minimal inhibitory concentration (MIC) was defined as the first clear tube after 7 days of incubation.

Assay of RNA synthesis. RNA synthesis was followed by measuring the incorporation of [^{3}H]uracil (0.5 μCi/ml) into the cold trichloroacetic acid-insoluble fraction of the mycobacteria according to the method of White et al. (8). The incorporation of [^{3}H]guanine (0.5 μCi/ml) into the 5% trichloroacetic acid-insoluble fraction of *H. capsulatum* and *B. dermatitidis* measured RNA synthesis in these organisms (5). The incorporation of [^{3}H]uracil into the mycobacteria and [^{3}H]guanine into the yeast was linear for at least 48 h. Each experiment was done in duplicate and repeated three times. The variability between experiments and duplicate values was less than 10%.

RESULTS

The indirect susceptibility tests with mycobacteria on 7H-11 agar are shown in Table 1. According to the criteria of mycobacterial susceptibility tests established by the CDC, *M. bovis* (BCG) was sensitive to all the drugs tested; *M. bovis* (BCG-R) was resistant to INH; *M. kansasii* was resistant to INH and ethambutol, and *M. intracellulare* was resistant to INH, rifampin, ethambutol, and streptomycin.

Figure 1 shows the effect of INH on RNA synthesis, as measured by [^{3}H]uracil uptake of each of the mycobacteria tested. *M. bovis* (BCG-R) was unaffected by 2.0 μg of INH per ml, and RNA synthesis was inhibited 20% by 100 μg of the drug per ml. INH at 20 μg/ml had no effect on RNA synthesis when tested on *M. kansasii* and *M. intracellulare*. In contrast, 0.5 μg of INH per ml inhibited RNA synthesis in *M. bovis* (BCG) by 50%, and at higher concentrations (2 and 100 μg/ml) there was a more dramatic effect. If 2.0 μg of INH per ml is defined as the test of susceptibility to INH, then inhibition of RNA synthesis in the susceptible strain occurred as early as 4 to 8 h after the start of incubation; no effect of this concentration on

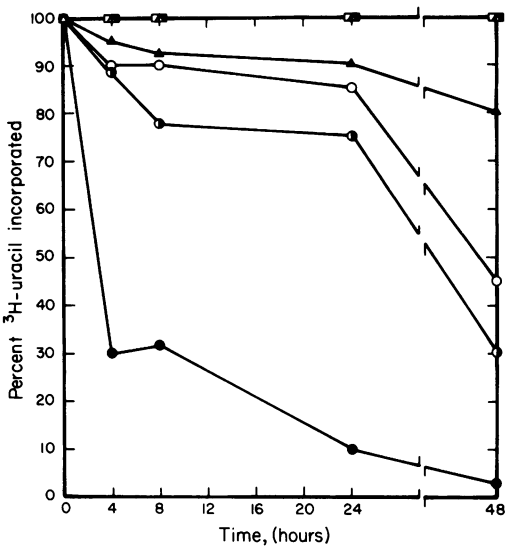

FIG. 1. *INH inhibition of [^{3}H]uracil incorporation into RNA of mycobacteria. Each point represents the average value from three experiments and is expressed as percent incorporation into organisms incubated without INH. The counts per minute per milliliter at 48 h for each of the control cultures are as follows: M. bovis (BCG), 13,220; M. bovis (BCG-R), 11,890; M. kansasii, 6,358; and M. intracellulare, 7,525. Symbols (with INH concentration in parentheses): ○, M. bovis (BCG) (0.5 μg/ml); ◑, M. bovis (BCG) (2.0 μg/ml); ●, M. bovis (BCG) (100 μg/ml); △, M. bovis (BCG-R) (2.0 μg/ml); ▲, M. bovis (BCG-R) (100 μg/ml); □, M. kanasasii (20 μg/ml); ■, M. intracellulare (20 μg/ml).*

TABLE 1. *Indirect susceptibility tests of mycobacteria on 7H-11 agar*

Drugs	No. of colonies							
	M. bovis (BCG)		*M. bovis* (BCG-R)		*M. kansasii*		*M. intracellulare*	
Inoculum dilution	10^{-2}	10^{-4}	10^{-2}	10^{-4}	10^{-2}	10^{-4}	10^{-2}	10^{-4}
Control	TNTC[a]	112	TNTC	68	TNTC	89	TNTC	78
INH (0.2 μg/ml)	0	0	TNTC	54	TNTC	78	TNTC	54
INH (1.0 μg/ml)	0	0	TNTC	32	TNTC	47	TNTC	93
Rifampin (0.1 μg/ml)	0	0	0	0	0	0	TNTC	101
Rifampin (5.0 μg/ml)	0	0	0	0	0	0	TNTC	39
Ethambutol (2.0 μg/ml)	0	0	0	0	TNTC	64	TNTC	83
Streptomycin (2.0 μg/ml)	0	0	0	0	0	0	TNTC	51
Streptomycin (10 μg/ml)	0	0	0	0	0	0	TNTC	38

[a] TNTC, Too numerous to count.

the resistant organisms was observed at this early stage.

By 8 h, RNA synthesis in mycobacteria susceptible to rifampin was inhibited by 25 to 50% in the presence of 0.1 and 1.0 μg of rifampin per ml, whereas *M. intracellulare*, the only rifampin-resistant strain, was unaffected by this concentration (Fig. 2).

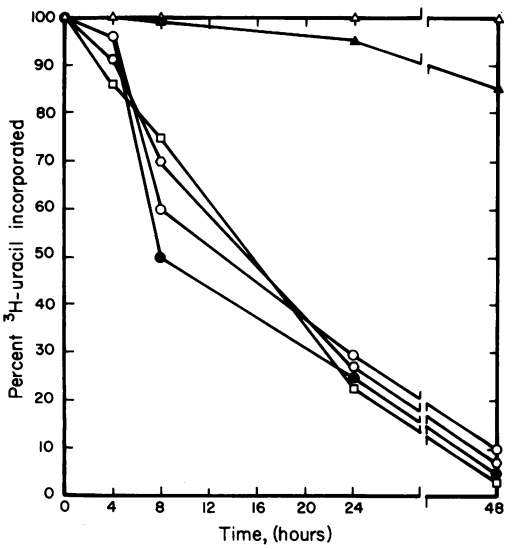

FIG. 2. *Rifampin inhibition of [³H]uracil incorporation into RNA of mycobacteria. Symbols (with rifampin concentration in parenthesis): △, M. intracellulare (1.0 μg/ml); ▲, M. intracellulare (5.0 μg/ml); □, M. bovis (BCG-R) (0.1 μg/ml); ○, M. kansasii (0.1 μg/ml); ○, M. bovis (BCG) (0.1 μg/ml); ●, M. bovis (BCG) (1.0 μg/ml).*

M. kansasii and *M. intracellulare* were resistant to ethambutol (Table 1), and RNA synthesis in these organisms was not inhibited by up to 10 μg of ethambutol per ml (Fig. 3), whereas by 8 h the susceptible strains *M. bovis* (BCG) and *M. bovis* (BCG-R) showed a 40 to 60% inhibition in the presence of 4.0 μg/ml.

RNA synthesis in *M. intracellulare*, the only streptomycin-resistant strain (Table 1), was unaffected by 10.0 μg of streptomycin per ml, whereas the streptomycin-susceptible strains *M. bovis* (BCG), *M. bovis* (BCG-R), and *M. kansasii* showed a 25 to 65% inhibition by 8 h and 60 to 85% inhibition at 48 h in the presence of 2.0 μg of streptomycin per ml (Fig. 4).

Figure 5 shows the effects of different concentrations of AmB and rifampin on RNA synthesis in our strain of *H. capsulatum*. A previous report from our laboratory (5) has shown that the MIC of AmB for the organism was 0.04 μg/ml and of rifampicin was 100 μg/ml. Both of these concentrations showed greater than 50% inhibition of RNA synthesis at 4 h.

Similar results are presented in Fig. 6 for *B. dermatitidis*. The MIC of AmB for this organism as determined by tube dilution was less than 0.01 μg/ml and for rifampin was 3.1 μg/ml. These drug concentrations showed greater than 50% inhibition of RNA synthesis in this fungus

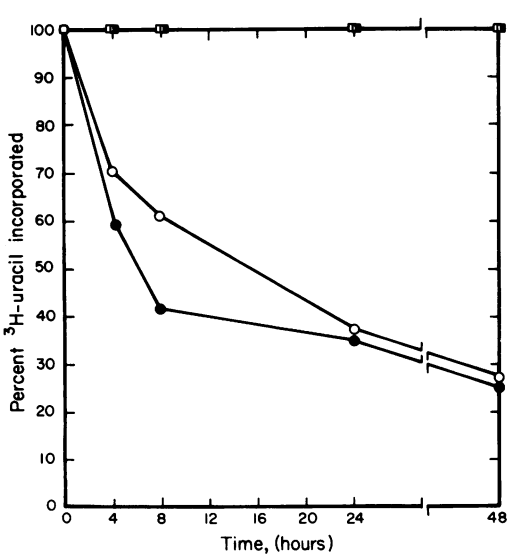

FIG. 3. *Ethambutol inhibition of [³H]uracil incorporation into RNA of mycobacteria. Symbols (with ethambutol concentration in parenthesis): □, M. kansasii (10.0 μg/ml); ■, M. intracellulare (10.0 μg/ml); ○, M. bovis (BCG) (4.0 μg/ml); ●, M. bovis (BCG-R) (4.0 μg/ml).*

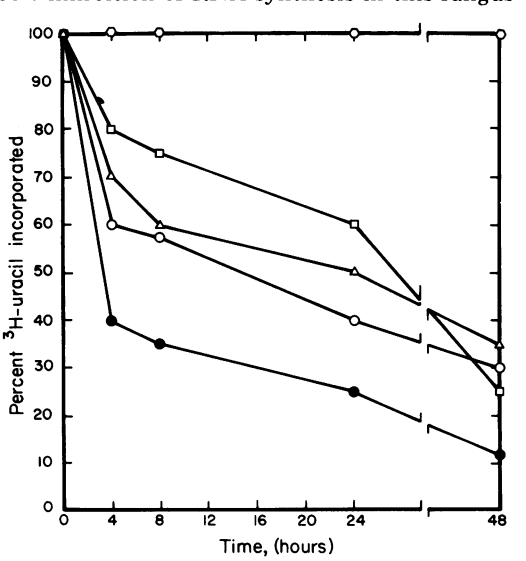

FIG. 4. *Streptomycin inhibition of [³H]uracil incorporation into RNA. Symbols (with streptomycin concentration in parentheses): ⬡, M. intracellulare (10.0 μg/ml); □, M. bovis (BCG-R) (2.0 μg/ml); △, M. kansasii (2.0 μg/ml); ○, M. bovis (BCG) (2.0 μg/ml); ●, M bovis (BCG) (10.0 μg/ml).*

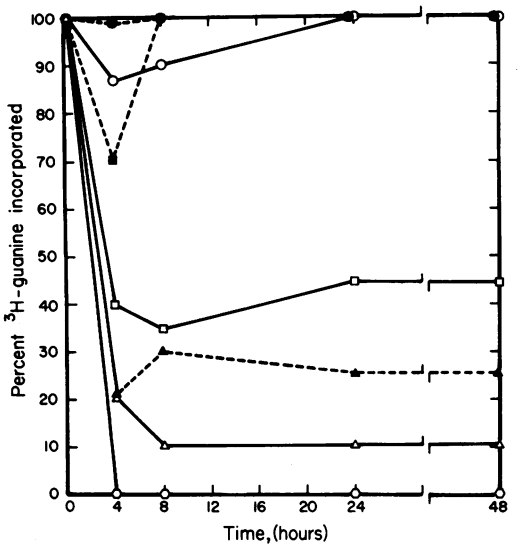

FIG. 5. *Amphotericin B and rifampin inhibition of [³H]guanine incorporation into the RNA of H. capsulatum. The control [³H]guanine incorporation at 48 h was 14,000 counts per min per ml. ○, AmB (0.02 μg/ml); □, AmB (0.04 μg/ml); △, AmB (0.08 μg/ml);◇, AmB (0.16 μg/ml); ●, rifampin (10 μg/ml); ■, rifampin (20 μg/ml); ▲, rifampin (100 μg/ml).*

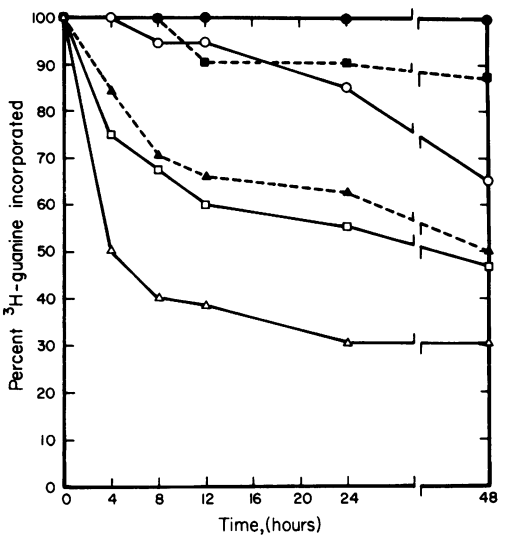

FIG. 6. *Amphotericin B and rifampin inhibition of [³H]guanine incorporation into the RNA of B. dermatitidis. The control [³H]guanine incorporation at 48 h was 10,000 counts per min per ml. ○, AmB (0.0025 μg/ml); □, AmB (0.005 μg/ml); △, AmB (0.01 μg/ml); ●, rifampin (1 μg/ml); ■, rifampin (2.0 μg/ml); ▲, rifampin (3.0 μg/ml).*

at 48 h. Both *H. capsulatum* and *B. dermatitidis* were resistant to sulfadiazine, polymyxin B, and 5-fluoro-cytosine by broth dilution susceptibility tests and by the radioisotope uptake method (data not shown).

DISCUSSION

[³H]uracil incorporation into the RNA of mycobacteria and [³H]guanine incorporation into the RNA of the yeast phase of fungi were used as measures of the viability of these organisms in the presence of several antibiotics. There appeared to be a direct correlation between the [³H]uracil incorporation and viability even in the presence of antibiotics such as AmB and streptomycin, which do not primarily affect RNA synthesis.

In every case those organisms susceptible to the antibiotics by traditional susceptibility testing showed an inhibition of RNA synthesis after short incubations in the presence of corresponding concentrations of the antibiotics, whereas those organisms resistant by the traditional methods did not show any decrease of RNA synthesis. By 48 h there was a 50% or greater inhibition of RNA synthesis in the susceptible strains. In most cases a smaller inhibition after 4 to 8 h of incubation in low concentrations of the antibiotic was also indicative of susceptibility.

There appeared to be an excellent correlation between the traditional susceptibility tests and the radioisotope method. Although the validity of a direct comparison of the MIC between the two methods has not been established and may be questionable because of the differences in time of incubation and parameters of growth, the consistency in results of the two methods we observed seems to indicate that the radioisotope method will be useful in determining antibiotic susceptibility and MIC. However, many more organisms will have to be studied to confirm these observations and to establish strict criteria for susceptibility to antibiotics and MIC. Also the sensitivity of the method to numbers of organisms in the incubation will have to be established. These more extensive studies are justified because of the rapidity of the radioisotope method when compared with the methods which depend on visible growth. In addition, the radioisotope method facilitates multiple drug screening and the determination of bacteriostatic and bactericidal effects, allows the evaluation of drug combinations for synergistic or antagonistic effects, and provides methods to determine the specific effects of the antibiotics on macromolecular synthesis.

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