APPLIED MICROBIOLOGY, June 1969, p. 861-865

Vol. 17, No. 6
Printed in U.S.A.

Antimycobacterial Activity of Rifampin Under In Vitro and Simulated In Vivo Conditions

K. D. STOTTMEIER, G. P. KUBICA AND C. L. WOODLEY
Mycobacteriology Unit, National Communicable Disease Center, Atlanta, Georgia 30333

Received for publication 12 March 1969

Minimal inhibitory concentrations of rifampin for different species of mycobacteria were determined in 7H-10 agar medium and Lowenstein-Jensen egg medium. When rifampin was incorporated into egg medium, approximately 90% of its activity was lost. The stability of rifampin was tested during storage at different temperatures and concentrations. When tested in agar medium, a combination of isoniazid (INH) and rifampin inhibited multiple drug-resistant strains of *Mycobacterium intracellulare*, but under simulated in vivo conditions the drugs did not eliminate these same organisms. Drug-resistant mutants of *M. intracellulare* multiplied during an 8-day period when exposed 10 hr daily to the INH-rifampin regimen. However, combinations of rifampin and INH reduced drug-resistant mycobacterial populations by 99%, an effect which could not be enhanced by the addition of either erythromycin, ethionamide, or cycloserine.

The antituberculous effect of rifampin in egg media, experimental animals, and tuberculous patients was first investigated in Europe by Clark and Wallace (2), Kradolfer (paper presented at the 19th International Tuberculosis Conference, Amsterdam, The Netherlands, 1967), Pines and co-workers (6), Verbist and Gyselen (8), and Gyselen and co-workers (3). Hobby confirmed the in vitro effect of rifampin on *Mycobacterium tuberculosis* and also tested isoniazid (INH)-rifampin combinations in tuberculous mice (4). The present study was undertaken to determine (i) the stability of aqueous solutions of rifampin on storage at different temperatures, (ii) the minimal inhibitory concentration (MIC) of rifampin for mycobacteria grown on Middlebrook 7H-10 agar medium, (iii) the antituberculous activity of rifampin in comparison to INH, and (iv) the antimicrobial effect of rifampin-INH combinations on *M. intracellulare* in 7H-10 medium and under simulated in vivo conditions of the Laboratory Model Man (7).

MATERIALS AND METHODS

The 62 strains of mycobacteria employed in this study included 21 strains of *M. tuberculosis*, 16 strains of *M. intracellulare*, and 5 strains each of *M. kansasii*, *M. terrae* complex, *M. fortuitum*, tap water scotochromogens, and scrofula scotochromogens. The drug susceptibility patterns of all strains were established for INH, dihydrostreptomycin sulfate, para-aminosalicylic acid (PAS), ethionamide, ethambutol, viomycin, kanamycin, cycloserine, and pyrazinamide by the proportion method of Canetti and coworkers (1). All mycobacterial strains other than tubercle bacilli and six strains of *M. tuberculosis* showed multiple drug resistance to three or more of the above mentioned drugs. The laboratory strain H37Rv and 14 other strains of *M. tuberculosis* were fully sensitive to all antituberculous drugs.

A 10-mg amount of rifampin (Pitman-Moore, Division of Dow Chemical Co., Indianapolis, Ind.) was solubilized in 2 ml of absolute ethyl alcohol, and 8 ml of distilled water was added to obtain a working stock solution of 1,000 μg/ml. Aqueous dilutions of rifampin could be made from this stock solution.

To test the stability of rifampin during storage, solutions of 10, 100, and 1,000 μg/ml of the drug were placed at −20, 4, and 25 C. After 4, 8, and 12 weeks of storage, the activity of the drug was determined by the Food and Drug Administration cylinder method (5) and a rifampin sensitive strain of *Staphylococcus aureus*.

The MIC of rifampin for *M. tuberculosis* was dedetermined in both 7H-10 agar medium and Lowenstein-Jensen (LJ) egg medium. The MIC of rifampin for mycobacteria other than *M. tuberculosis* was determined in 7H-10 medium only. Twelve different concentrations of rifampin ranging from 0.05 to 7.5 μg/ml were incorporated into either 7H-10 agar medium or LJ medium. In the case of 7H-10 medium, the various drug-containing media and drug-free controls were dispensed in 5-ml amounts into quadrants of sectioned plastic petri plates; comparable batches of LJ medium were dispensed in 5-ml amounts into screw-capped glass tubes (16 by 125 mm). The tubes containing egg medium were inspissated in a slanting position for 45 min at 85 C.

Rifampin in a concentration of 1 μg/ml in 7H-10

agar was also compared with paper discs containing 5 μg of rifampin, by the method of Wayne and Krasnow (9). Drug discs were placed in the center of empty quadrants of plastic petri dishes, and 5 ml of 7H-10 agar was layered over each drug-containing disc. Both batches of plates were kept for 24 hr at room temperature and 48 hr at 4 C prior to their inoculation with two strains each of *M. kansasii*, *M. intracellulare*, both scrofula and tap water scotochromogens, and *M. terrae*. Three different sizes of bacterial inocula were tested for each strain.

In 7H-10 agar medium, the antituberculous effect of five concentrations of rifampin, ranging from 0.05 to 0.25 μg/ml, was compared with the effect of the same concentrations of INH. Fifteen strains of *M. tuberculosis* from untreated tuberculous patients were tested against each of the five concentrations of INH and rifampin in 7H-10 agar. Sixteen strains of *M. intracellulare* were tested in 7H-10 agar against (i) 1 and 5 μg of rifampin per ml; (ii) four different combinations of 1 or 5 μg per ml each of INH plus rifampin; and (iii) six different combinations of either 1 or 5 μg of rifampin plus 5 μg of INH per ml, supplemented with either 5 μg of erythromycin or ethionamide per ml, or 20 μg of cycloserine per ml. The susceptibility of *M. intracellulare* to different combinations of two or three drugs was tested with two different sizes of inoculum by the proportion method of Canetti and co-workers (1).

The antimicrobial effect of INH-rifampin combinations under simulated in vivo conditions was studied in Laboratory Model Man experiments (7). One ml of 5-day-old 7H-9 broth cultures of two strains of *M. intracellulare* was aseptically added to a series of dialysis sacs having a pore size of 4.8 nm. The sac containing broth cultures were then exposed to different drug combinations and concentrations for 10 hr a day for 10 days.

For 10 days, each culture bag was alternately placed for 10 hr into 200 ml of 7H-9 broth containing INH-rifampin and for 14 hr into 200 ml of drug-free broth. The dialysis of the drugs into and out of the culture-containing bag into the surrounding reservoir simulated the absorption and excretion of INH and rifampin in man. The full drug concentration, i.e. the peak level, was achieved in the culture bag 4 hr after placing it in drug-containing broth. The level was maintained for 6 hr, then the culture was transferred to drug-free medium, and within 4 hr all detectable drug had disappeared. The effect of the drug combinations was expressed quantitatively by determining the numbers of surviving organisms. Beginning on the first day of the experiment, and then every other day for a 10-day period, samples were taken from each culture bag for a plate count on 7H-10 agar medium.

Organisms which survived 10 days of antimicrobial treatment with a given drug combination were tested on 7H-10 agar containing the same drugs as the treatment regimen. All cultures on 7H-10 agar medium were incubated at 37 C in an atmosphere of 10% CO_2, whereas other experimental cultures were incubated in regular incubators at 37 C.

RESULTS

Solutions of 1,000 μg of rifampin per ml may be kept for 12 weeks at refrigerator (4 C) or deep-freeze (−20 C) temperature without losing more than 9% of their activity (Table 1). Similar observations were made by storing 100 to 1,000 μg of rifampin per ml for 8 weeks at these temperatures, or for 4 weeks at 25 C. Storing rifampin in concentrations lower than 100 μg/ml increases the rate of deterioration. Of the 10 μg/ml drug concentrations, 6 to 15% were lost after only 4 weeks at temperatures from −20 C to 25 C, 7 to 31% were lost after 8 weeks, and 20 to 48% were lost after 12 weeks. The MIC range of rifampin for *M. tuberculosis* is shown in Fig. 1. Each of the 15 strains tested is represented by a dot on the scattergraph; the location of each dot is dependent upon the inhibitory concentra-

TABLE 1. *Stability of aqueous solutions of rifampin stored for 3 months at −20 C, 4 C, and 25 C*

Storage temp	Rifampin conc (μg/ml)	Rifampin activity remaining after		
		4 Weeks	8 Weeks	12 Weeks
C		%	%	%
−20	1,000	99.0	93.0	91.0
	100	97.0	93.0	91.0
	10	94.5	93.0	88.5
4	1,000	98.0	95.0	92.0
	100	98.0	93.5	88.5
	10	96.5	87.0	79.5
25	1,000	92.5	89.5	75.4
	100	92.5	88.0	75.0
	10	85.5	69.0	52.0

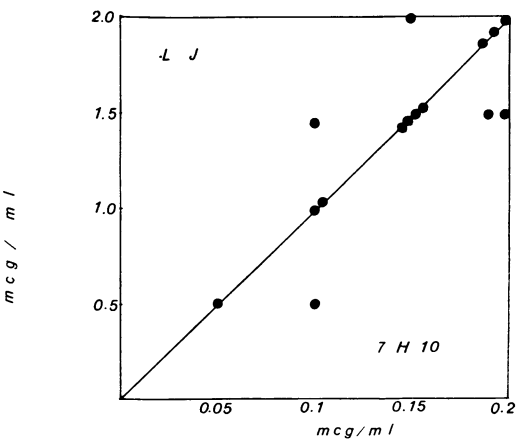

FIG. 1. *MIC of rifampin for 15 strains of M. tuberculosis in 7H-10 and LJ medium.*

tion of rifampin in both 7H-10 medium (abscissa) and LJ medium (ordinate). All strains were inhibited by 0.05 to 0.2 μg of rifampin per ml of 7H-10 medium or by 0.5 to 2.0 μg of rifampin per ml of LJ medium, indicating a 90% inactivation of the drug in egg medium. This inactivation was not totally due to the inspissation since only 11 and 20% of an aqueous solution of 1 μg of rifampin per ml was lost after boiling for 1 hr or autoclaving (121 C) for 15 min.

The MIC of rifampin in 7H-10 medium for mycobacteria other than *M. tuberculosis* ranges from 0.1 to more than 5.0 μg/ml (Fig. 2). Strains of *M. kansasii* were sensitive to 0.2 to 0.5 μg/ml; scrofula scotochromogens (indicated as "scrof") were sensitive to 0.1 to 2.0 μg/ml; *M. terrae* strains were sensitive to 0.15 to 2.0 μg/ml; tap water scotochromogens ("aquae") were sensitive to 0.5 to 5.0 μg/ml; and five strains of *M. fortuitum* were inhibited by rifampin concentrations ranging from 2.0 to more than 5.0 μg/ml. Sixteen strains of *M. intracellulare* were resistant to 1 μg of rifampin per ml, whereas 5 of these 16 strains were inhibited by 5 μg/ml (Table 2).

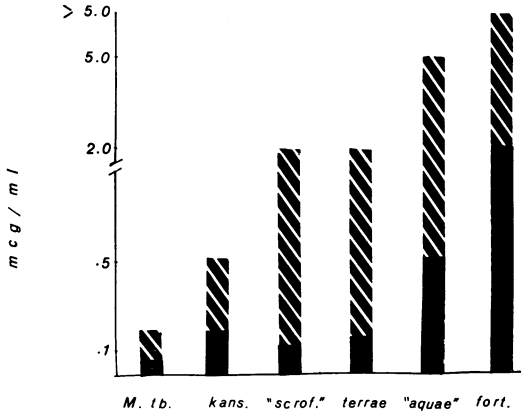

FIG. 2. *Ranges of MIC of rifampin for different species of mycobacteria. Solid bars represent lowest MIC; striped bars depict observed range of MIC levels.*

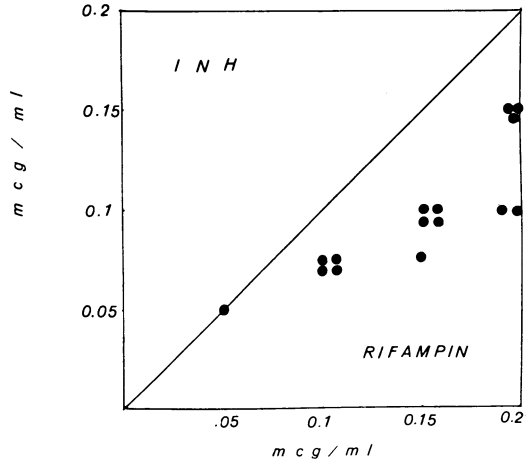

FIG. 3. *MIC of rifampin and INH for 15 strains of M. tuberculosis.*

TABLE 2. *Susceptibilities of 16 strains of M. intracellulare to rifampin alone or in combinations with other antituberculous drugs in 7H-10 agar medium*[a]

No. of strains	Drug combinations investigated					
	R5	R1 + I1	R5 + I1	R1 + I5	R5 + I5	R5 + I5 + C20/ Er5/Et5
2	Res	Res	Res	Res	Res	Res
6	Res	Res	Res	Sen	Sen	Sen
1	Res	Res	Sen	Sen	Sen	Sen
2	Sen	Res	Sen	Sen	Sen	Sen
2	Res	Sen	Sen	Sen	Sen	Sen
3	Sen	Sen	Sen	Sen	Sen	Sen

[a] Abbreviations: R1, 1 μg of rifampin per ml; R5, 5 μg of rifampin per ml; I1, 1 μg of INH per ml; I5, 5 μg of INH per ml; C20, 20 μg of cycloserine per ml; Er5, 5 μg of erythromycin per ml; Et5, 5 μg of ethionamide per ml; sen, no mycobacterial growth; res, more than 1% of the control growth.

Comparative susceptibility tests in 7H-10 medium with either rifampin contained in discs or incorporated into the agar revealed disagreement in only 1 out of 10 strains tested. A scotochromogenic strain did not grow on medium in which 1 μg of rifampin was incorporated per ml; however, 100 colonies grew on medium containing the appropriate rifampin drug disc.

The antituberculous activity of INH and rifampin are compared in another scattergraph (Fig. 3). Strains of drug sensitive tubercle bacilli are represented by dots that are located according to their susceptibility to INH (ordinate) and rifampin (abscissa). Fourteen strains were inhibited by 0.075 to 0.15 μg of INH but by 0.1 to 0.2 μg of rifampin per milliliter, thus indicating that the MIC of rifampin are 25 to 50% higher than those of INH.

The antimycobacterial activity of INH-rifampin combinations against 16 strains of *M. intracellulare* that were resistant to 1 μg of rifampin per ml was studied (Table 2). The addition of 1 μg of INH per ml to a rifampin concentration of 1 or 5 μg/ml inhibited the growth of 5 and 8

TABLE 3. *Antimicrobial effect of combinations of rifampin and INH on Mycobacterium intracellulare in the Laboratory Model Man*

Combinations of drugs	No. of viable units isolated after treatment for (days)					
	0	2	4	6	8	10
Control	10^3	10^4	5×10^4	10^5	2×10^5	5×10^5
R5 + I1	10^3	10^4	10^4	10^3	10^3	10^3
R1 + I5	10^3	10^4	$<10^4$	10^2	$<10^2$	$>10^2$
R5 + I5	10^3	10^4	10^3	10^2	$<10^2$	$>10^2$

strains, respectively, of *M. intracellulare* on 7H-10 agar. Fourteen strains were inhibited by a combination of 1 or 5 μg of rifampin plus 5 μg of INH per ml.

Broth cultures of *M. intracellulare* were exposed for 10 hr daily to three different combinations of INH-rifampin. By simulating the in vivo conditions of absorption and excretion of drugs, the combination of 5 μg of rifampin and 1 μg of INH per ml exerted a bacteriostatic effect on a population of *M. intracellulare*; however, an initial multiplication of the organisms was observed during the first 2 days of treatment (Table 3). The number of organisms in an untreated control increased within 10 days from 10^3 to 5×10^5. Combinations of either 1 or 5 μg of rifampin plus 5 μg of INH per ml decreased the population to less than 100 organisms per ml within 8 days of treatment (a 99% reduction). Rifampin-INH resistant mutants then began to multiply to $> 10^2$ between the 8th and 10th day, indicating the necessity of a third drug for the elimination of the organisms (Table 3). However, neither 5 μg/ml concentrations of erythromycin or ethionamide nor 20 μg/ml of cycloserine enhanced the antimicrobial effect of the INH-rifampin combinations in vitro (Table 2).

DISCUSSION

The stability of rifampin in aqueous solutions is similar to that of aqueous solutions of dihydrostreptomycin sulfate. Both drugs lose less than 10% of their antituberculous potency if stored at 100 to 1,000 μg/ml concentrations at −20 C for up to 3 months. However, streptomycin remains stabile in aqueous solutions (*p*H 3 to 7) up to 3 months at 25 C (5), whereas solutions of 10 to 1,000 μg of rifampin per ml lost 25 to 48% of their activity under these conditions. When rifampin was incorporated into an inspissated egg medium such as Lowenstein-Jensen, there was approximately a 90% loss of activity. Only 11 to 20% of rifampin activity was lost by boiling or autoclaving in aqueous solutions. Therefore, rifampin, like streptomycin, seems to be inactivated by the phospholipids of the egg yolk (5).

Verbist and Gyselen (8) isolated one rifampin-resistant colony of *M. tuberculosis* per 1,000 to 10,000 viable organisms inoculated onto LJ medium containing 2.5 μg of rifampin per ml prior to inspissation. Using the inocula of 50 to 500 viable units, we isolated no resistant mutants from 15 strains of *M. tuberculosis* which had been inoculated onto 7H-10 medium containing 0.2 μg of rifampin per ml. The MIC of rifampin for tubercle bacilli were reported to vary from 0.02 to 0.31 μg/ml in 2 liquid media (4). According to G. L. Hobby (paper presented at 28th Veterans Administration Armed Forces Conference, Cleveland, Ohio, 1969), the MIC of rifampin for *M. tuberculosis* in 7H-10 agar medium ranges from less than 0.2 to 1.0 μg/ml.

The relatively low MIC of rifampin for *M. kansasii* and scrofula scotochromogens (Fig. 2) suggests this drug for the treatment of these mycobacterioses in man. *M. kansasii* strains studied by Hobby were sensitive to 0.02 to 10.0 μg of rifampin per ml in liquid media (4). Careful susceptibility testing of *M. kansasii* and scrofula scotochromogens revealed some of these strains to be as sensitive to rifampin as was *M. tuberculosis*, although others were only inhibited by 2 μg/ml or more. More than 5 μg of rifampin per ml was required for the inhibition of *M. intracellulare* and *M. fortuitum*. However, 14 out of 16 strains of *M. intracellulare* were inhibited by 1 μg of rifampin combined with 5 μg of INH per ml of 7H-10 medium. Two of the 14 strains were tested against INH-rifampin combinations under simulated in vivo conditions and both strains eventually survived the daily 10-hr drug treatment by virtue of selection of INH-rifampin-resistant mutants. For the elimination of multiple drug-resistant populations of *M. intracellulare*, a two-drug combination of INH-rifampin does not seem to be sufficient; and the addition of neither cycloserine, ethionamide, nor erythromycin to the 2-drug regimen enhanced the antimicrobial effect.

These in vitro studies demonstrate that rifampin has an antituberculous activity comparable to that of INH and streptomycin. Further, these

tests indicate that rifampin should be a valuable adjunct to the drug regimens used to treat tuberculosis and certain other mycobacterioses.

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