Bacteriologic Studies of Rifampin, a New Semisynthetic Antibiotic

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Rifampin is a semisynthetic N-methyl piperizine derivative of the antibiotic rifamycin SV produced by *Streptomyces mediterranei*. It is of particular interest because of its wide antibacterial spectrum against gram-positive and gram-negative bacteria as well as *Mycobacterium tuberculosis* [1–4]. It is an orange-red powder with a molecular weight of 822 and has the structure shown in figure 1. Clinical evaluation is now under way.

This report will deal with the in vitro activity of this antibiotic against staphylococci, streptococci, and enteric bacteria. Particular emphasis will be placed on the problem of emergence of resistant strains and the effect of inoculum size on assay by dilution methods. Preliminary data are also presented on the recovery of the drug in the urine of healthy volunteers.

Methods

Bacterial strains. Subcultures of staphylococci isolated from cases of human infection were kindly provided by the Laboratory Branch of the National Communicable Disease Center. Most of these were characterized by phage type and provided a representative sample of strains encountered in this country. Group A streptococci (identified by beta hemolysis on sheep blood and sensitivity to bacitracin) were obtained from the clinical laboratory of the University of Virginia Hospital. Enteric bacteria were isolated from patients with urinary tract infections. All strains were subcultured on suitable semisolid media, and a single colony was picked and used in subsequent studies. Bacteria were maintained by serial passage in trypticase soy broth.

Received for publication July 29, 1968.

This work was supported in part by training grant AI-00266 from the U.S. Public Health Service, and by the CIBA Pharmaceutical Co., Summit, N. J.

The authors wish to thank Mrs. Ann Bugg, Louise Moore, and Virginia Breeden for their able technical assistance.

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Sensitivity studies. These were conducted by the twofold broth dilution, streak plate, and disk methods. The dilution method was standardized to provide 0.5 ml of the appropriate dilution of antibiotic, to which was added 0.5 ml of bacteria inoculum. The mixture was then incubated overnight at 37 C. Nutrient broth was used for staphylococci and enteric bacteria; trypticase soy broth containing 3% human O blood was used for streptococci. Plate dilutions were made by adding 1 ml of antibiotic solutions to a plate together with 9 ml of molten nutrient agar or, in the case of streptococci, with 4% sheep blood in blood agar base. Plates were streaked with an overnight culture of each strain taken up in a plastic 1-ml pipette. This procedure delivered approximately 1×10^6 colony-forming units as the tip of the pipette was drawn across the plate and moistened the surface.

Resistant strains were isolated by adding 1 ml of an overnight broth culture to a plate containing 1 ml of antibiotic dilution to which were added 8 ml of molten agar. Strains growing on these plates were picked, subcultured, and tested for sensitivity on rifampin streak plates and for sensitivity to other antibiotics by the disk sensitivity method. The number of organisms in the initial culture was determined by serial plate dilution.

The activity of the drug against staphylococci was assessed in growth curve experiments by adding various inocula of overnight cultures to nutrient broth containing 1 μg of rifampin per milliliter and to control tubes without drug. These tubes were incubated at 37 C, and samples were plated at intervals to permit enumeration of viable organisms. Plate sensitivity tests were performed prior to and at completion of 48 hr of incubation with antibiotics. In addition, 1 ml of each culture, after 72 hr of incubation with the drug or in control tubes, was added to a plate containing a final concentration of 10 μg of rifampin per milliliter of agar. The number of colonies growing on the plates was compared to the number of viable organisms in each culture,

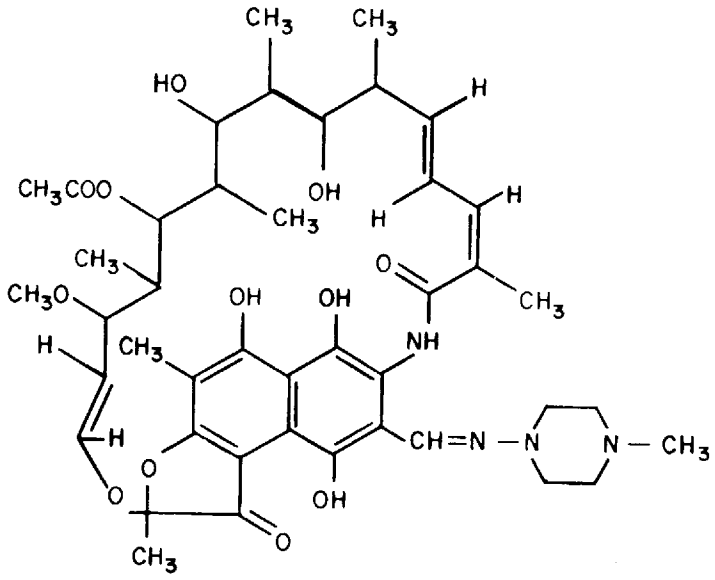

Figure 1. Chemical structure of rifampin

Table 1. Effect of inoculum size on minimum inhibiting concentrations of rifampin against 8 strains of staphylococci in nutrient broth

Strain		Low inoculum		High inoculum		Skip tubes*
Strain	Phage type	No. × 10^3	MIC	No. × 10^6	MIC	
93	53/86	4.5	1.25	7.0	> 5	None
1423	Untyped	0.8	0.0013	6.0	0.6	8, 9, 10, 12
1214	80/81	0.6	0.0013	3.9	5	3, 4
2553	Untyp.	0.4	0.003	2.0	5	3, 4, 7, 8
2504	29/52	0.3	0.0003	2.8	> 5	None
1821	36/55/71	4.5	0.0013	9.0	2.5	5, 6, 8, 12
172	29/79	1.5	0.0013	3.0	5	3, 11
2099	Untyped	0.2	0.0006	0.8	5	3, 4, 5, 8

* The test was done using twofold dilutions starting at 5 μg/milliliter; skip tubes were scattered tubes showing no growth at various points in the dilution series. The MIC, reported in microgram per milliliter, was measured at the point above which all tubes were clear.

as assessed by dilution in plates free of the drug. This provided information on the relative number of resistant colonies in each culture.

Results

Activity against staphylococci. Two hundred strains of *Staphylococcus aureus* were tested for sensitivity to penicillin G and rifampin by the streak plate method. Sixty-nine of these were resistant to more than 1 μg of penicillin G per milliliter. All but 3 of the strains tested, however, were highly sensitive to rifampin at concentrations of 0.002 to 0.008 μg/milliliter.

Tube dilution tests produced varying results, depending upon inoculum size. Small inocula, containing between 10^2 and 10^3 colony-forming units, revealed the staphylococci to be highly sensitive to rifampin, with the exception of 1 strain which was known to be moderately resistant on streak plates (table 1). Large inocula, containing between 10^5 and 10^6 colony-forming units demonstrated that most strains show only slight sensitivity to the drug and that "skip" tubes occur frequently in the dilution series (table 1). (A skip tube is defined as a tube in a dilution series which reveals no growth even though tubes containing higher concentrations of drug fail to inhibit the test strain.)

In view of these results, resistant variants in the original inoculum were sought by plating overnight cultures of 20 strains of staphylococci, originally shown to be highly sensitive to rifampin, in the presence of 10 μg of the drug per milli-

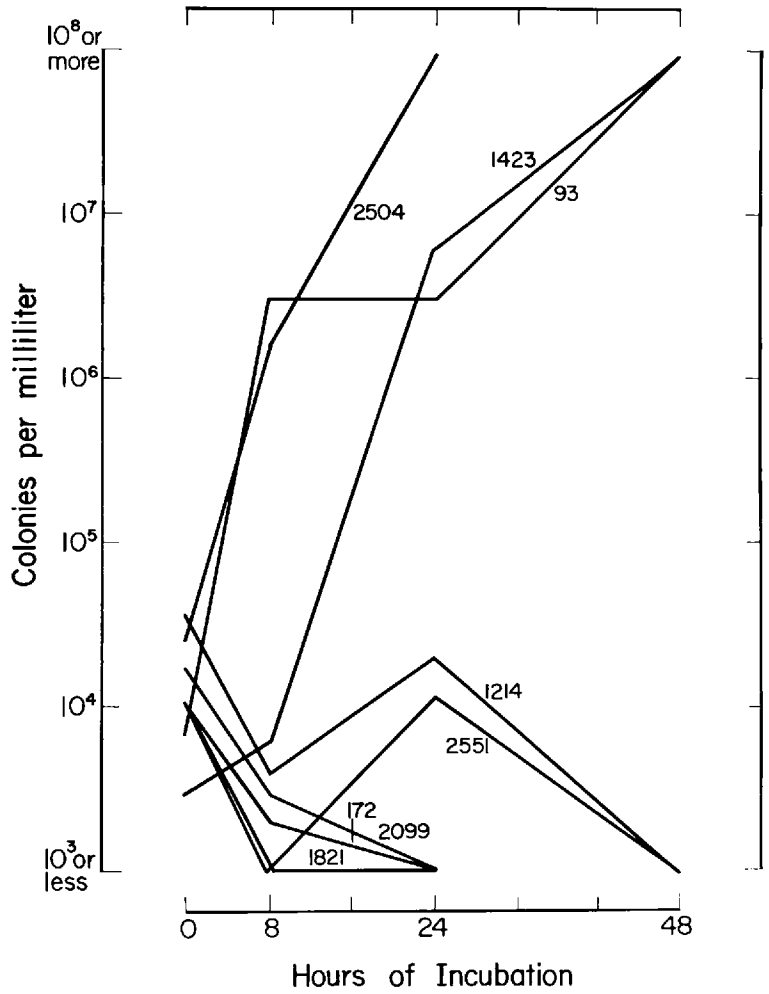

Figure 2. Effect of rifampin, 1 μg/milliliter, on small inocula of 8 strains of *Straphylococcus aureus* grown in nutrient broth

liter of agar. The frequency of resistant colonies varied from 5×10^{-6} to 7×10^{-8} with an average of 1 resistant colony for 1.4×10^{-8} colony-forming units. Single isolates of each plate were picked, subcultured, and found to be still resistant to more than 10 μg of the drug per milliliter of agar on streak plates.

Cross-sensitivity studies with tetracycline, chloramphenicol, erythromycin, novobiocin, methicillin, and penicillin G were conducted with each of the 20 strains prior to exposure to rifampin and with subcultures of the resistant variants. Zone diameters (employing 3 replicate disks per plate) were identical for rifampin-sensitive and rifampin-resistant strains, indicating that no cross-resistance to the other antibiotics had occurred.

Further evidence of rapid emergence of resistant variants was obtained in subsequent experiments (figures 2 and 3). In these studies, small and large inocula were exposed to 1 μg of rifampin per milliliter of broth. Killing was observed with 5 of 8 strains in which small inocula were used (figure 2). With large inocula, killing was also observed with 5 strains, but 3 grew to high bacterial numbers after 8 hr. Although rifampin-resistant variants in 2 of these 3 grew slowly on initial subculture, they were coagulase- and mannitol-positive and retained their resistance to the drug.

Activity against enteric bacteria. Tube dilution studies were conducted with Proteus, *Escherichia coli*, and nonmotile Klebsiella strains, employing a 1×10^{-4} dilution of an overnight culture as inoculum (figure 4). Proteus strains (all but 1 of which were indol-negative) were most sensitive, followed by *E. coli*, and the Klebsiella. The minimum inhibiting concentrations in these studies are based on 24-hr end points. Incubation for another 24 hr reduced these values, in most cases, by 1 tube (twofold). The frequency of variants

of *E. coli* resistant to more than 100 μg/milliliter was determined by exposure of 1 ml of an overnight culture of each of 10 strains to this concentration of drug in pour plates. Variants were noted in each culture at frequencies of 1-5 × 10^{-7} of the original culture population.

Activity against streptococci. Five strains of group A streptococci were found to be sensitive to 0.015 μg/milliliter of rifampin in tube dilution tests when small inocula of 4-29 × 10^3 colony-forming units were used. A hundredfold larger inoculum required fourfold more drug to inhibit growth at 24 hr. Further loss of activity by 1 tube (twofold) occurred after 24 more hours of incubation. A small number of resistant variants was found in plating experiments as described above, using cultures previously not exposed to the drug. These variants initially grew only on plates containing 0.5 μg/milliliter or less of rifampin, but on subculture some grew on plates containing 100 μg/milliliter. These resistant strains continued to hemolyse sheep blood agar and were inhibited by bacitracin.

The exact frequency of variants of streptococci resistant to rifampin was difficult to assess since the population density used in these experiments did not exceed 2-3 × 10^8 colony-forming units. It is estimated, however, that such variants, resistant to 1 μg of rifampin per milliliter or less, were present at a frequency of about 1 resistant colony per 1 × 10^{-7} colony-forming units.

Recovery of the drug in urine. The activity of rifampin against enteric bacteria, particularly strains of Proteus, prompted a study of the recovery of the drug in urine. Five healthy young male volunteers in the fasting state were given 300 mg in capsules, and urine samples were collected at intervals (figure 5). Specimens were assayed (under code) by the cup-plate method using *Sarcina lutea*, courtesy of CIBA Pharmaceu-

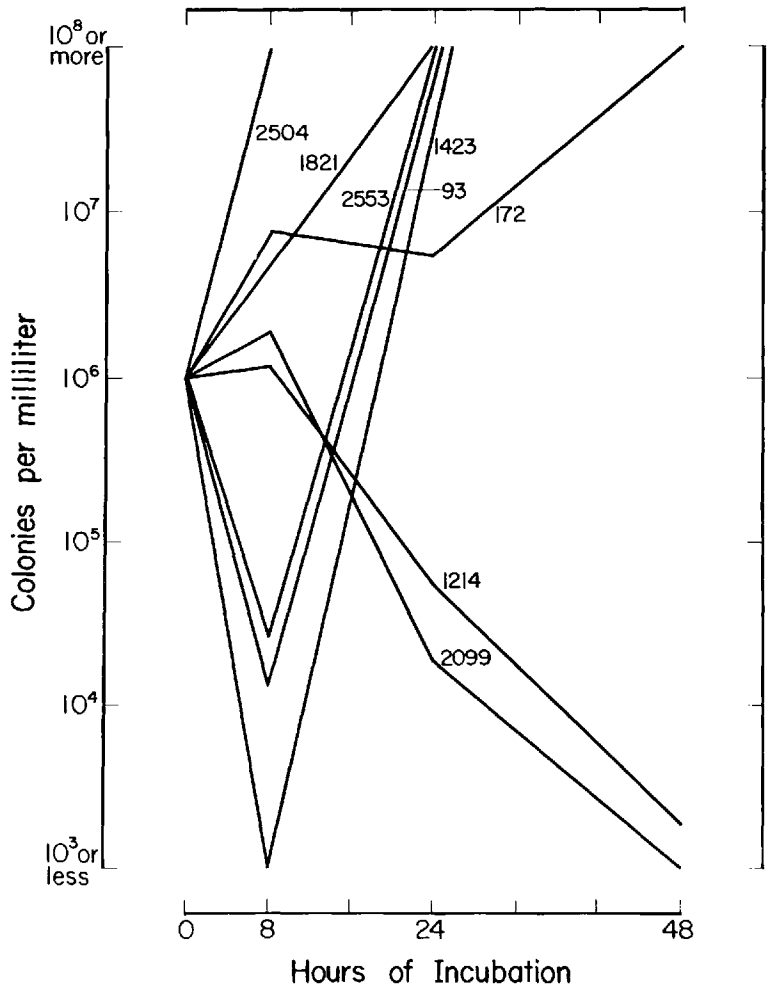

Figure 3. Effect of rifampin, 1 μg/milliliter, on large inocula of 8 strains of *Straphylococcus aureus* grown in nutrient broth

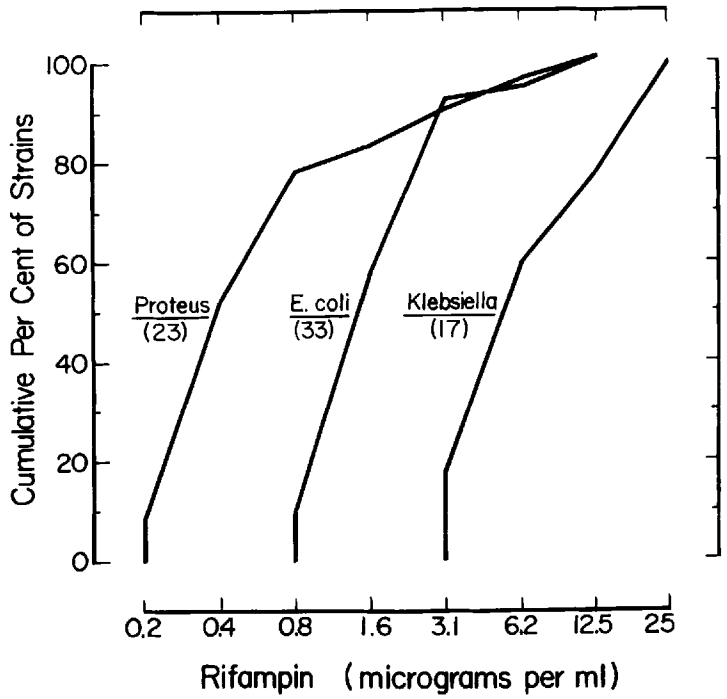

Figure 4. Minimum inhibiting concentrations of rifampin in nutrient broth tests against 3 species of enteric bacteria as read at 24 hr of incubation.

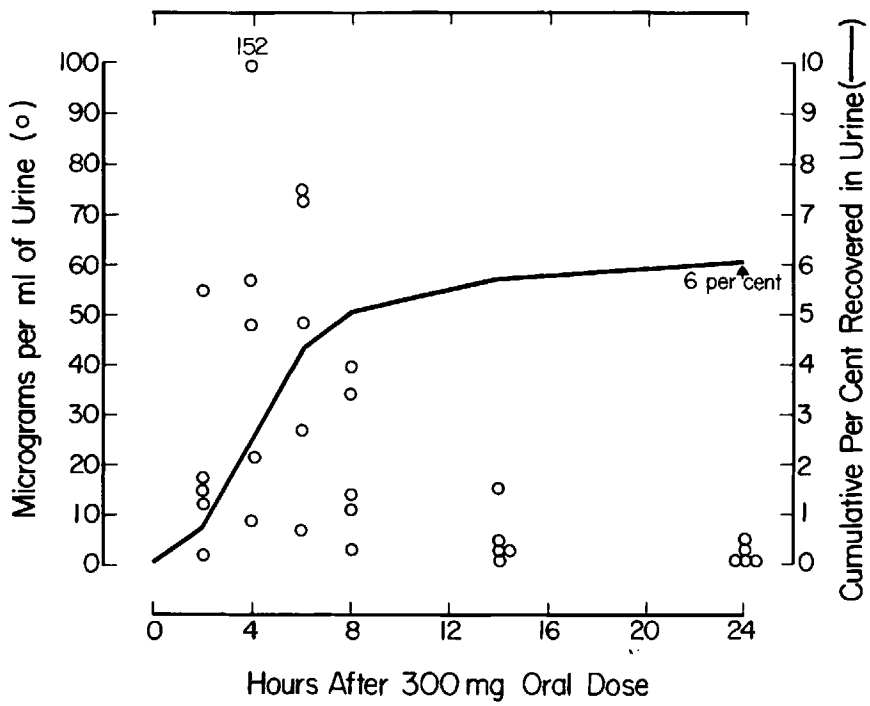

Figure 5. Recovery of rifampin in the urine of healthy volunteers with good renal function following a 300 mg oral dose taken while fasting.

tical Co. of Summit, New Jersey. Six per cent of the oral dose was recovered in the urine, a figure consistent with previous pharmacological studies [2]. Peak concentrations were obtained between 4 and 8 hr and were well within a range to affect Proteus, *E. coli*, and most strains of Klebsiella.

Tube dilution studies were conducted in our

laboratory of specimens of serum and urine obtained from volunteers, using a staphylococcus and a streptococcus as assay strains. The results of these studies varied considerably when they were repeated and did not correlate well with cup-plate assays. For example, an assay of urine specimens by the twofold dilution method, using Staphylococcus 209P, gave results only one-tenth as high as those reported by the cup-plate method. This was probably due to the important effect of inoculum size and time of incubation on activity of the drug. For this reason, results of such studies must be interpreted with caution.

Discussion and summary

The bacteriologic studies of rifampin reported here demonstrate that this antibiotic has promising activity against staphylococci, group A streptococci, and certain enteric bacteria, particularly Proteus. The presence of resistant variants, found in all cultures of staphylococci and *Escherichia coli* tested, and in most cultures of group A streptococci as well, probably accounts for the problems encountered in the in vitro assessment of the drug by streak or tube dilution methods. Assay methods using streak plates or small inocula in tube dilution tests may be misleading since they do not reflect the potential rapid emergence of resistant variants in each culture. Rifampin shows promise in treatment of some forms of urinary infection and may prove to be an adjunct in the therapy of staphylococcal and streptococcal infections. It would seem wise, however, to recall the experience with rapid emergence of resistant strains of staphylococci to erythromycin and novobiocin and to use this new drug together with another agent to which the organism is sensitive [5]. Further, it must be shown that drug antagonism does not occur in combination therapy of this type [6].

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