A Rapid Antibacterial Test for Mycobacteria Using a Reverse T Type of Tube in Rocked Culture

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SUMMARY: Antibacterial tests with mycobacteria were carried out in rocked $\perp$ tubes (Monod). The medium used was Sauton medium + 1 % (w/v) Tween 80. It took 48 hr. to get a reproducible value for the ID₅₀ dose (50 % inhibition dose) with tubercle bacilli of human type, and 8 hr. with the avian type.

Antibacterial tests with mycobacteria may take a long time, for instance several weeks in the case of human tubercle bacilli. When an acid-fast saprophyte is used for shortening the time for the test, the results obtained sometimes cannot be translated directly as measures of the effect of the drug on human type organisms. We reported (Aoyagi & Mizuno, 1959) that various strains of mycobacteria could be grown in a non-protein medium in rocked $\perp$ tubes and that mycobacteria could be treated conventionally for the determination of growth curves by observations of the optical density (OD) of cultures, with the use of suitable calibration curves to give absolute measures of amounts of growth. The present paper deals with an antibacterial test for mycobacteria with this type of rocked culture, estimating the ID₅₀ dose (50 % inhibition dose) by the method of Treffers (1956) where two or three dilutions of drug are sufficient, and observing the growth of bacteria in the logarithmic phase. The ID₅₀ dose can be read from graphs obtained by taking drug concentration plotted on a logarithmic scale as abscissa and probit of % inhibition as ordinate.

METHODS

Organisms. *Mycobacterium tuberculosis* var. *hominis* strain H37Rv and *M. avium* strain A-62 were used; details are given in the preceding paper (Aoyagi & Mizuno, 1959).

Medium. This was prepared as follows: asparagine, 4.0 g.; citric acid, 2.0 g.; K_2HPO_4 , 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; ferric ammonium citrate, 0.05 g.; dissolved in 930 ml. distilled water, adjusted to pH 7.2 and autoclaved. Separately, 40 % (w/w) glucose solution was prepared and sterilized at 100° for 30 min. on three successive days. To each 9.3 ml. of basal medium was added 0.5 ml. glucose solution. Tween 80 solution (0.2 ml. 50 %, w/v; autoclaved) was then added to give a final Tween 80 concentration of 1.0 %. Other details were given in the preceding paper.

Incubation and inoculum. The same procedure as described previously (Aoyagi & Mizuno, 1959) was used.

Drugs test. Crystalline dihydrostreptomycin sulphate (Meiji); viomycin (Parke, Davis); sodium *p*-aminosalicylate (Tanabe); Tibion (Takeda); pyra-

zinamide (Sankyo); levulinic acid oxime (Institute of Applied Microbiology, University of Tokyo); isonicotinic acid hydrazide (Tanabe); angstromycin (Institute of Applied Microbiology, University of Tokyo); *N*-hydroxy-2-pyridine thione (Tanabe); pyridomycin (Sankyo).

Determination of ID₅₀ dose. According to Treffers (1956) the ID₅₀ dose of a drug may be determined by intercepting a 50 % inhibition, taking as abscissa drug concentration plotted on a logarithmic scale and as ordinate probit of % inhibition. The % inhibition was determined by comparing the growth of the organisms with and without drug during the logarithmic phase of growth by observations of the optical density of the cultures in the rocked $\perp$ tubes. Inhibitions of more than 90 % and less than 10 % were not used for the ID₅₀ dose determination.

RESULTS

ID₅₀ of several drugs against tubercle bacilli

Figure 1*a* shows the normal response curve for *Mycobacterium avium* strain A-62 with dihydrostreptomycin and viomycin. The time needed to determine the degree of growth inhibition was 8 hr. The same procedure was followed for *M. tuberculosis* strain H37Rv (Fig. 1*b*), the time needed to obtain the response curve being 48 hr. Other antituberculous drugs were tested by this procedure to estimate the ID₅₀ dose against *M. avium* A-62 and *M. tuberculosis* H37Rv; the results are shown in Table 1.

Fig. 1*a*. The relationship between the drug concentration (plotted on logarithmic scale) and the probit of % inhibition of bacterial growth. ○—○, dihydrostreptomycin; and ●—●, viomycin. The ID₅₀ dose can be obtained from this linear relation at the ordinate intercept of a 50% end point. The slopes for viomycin and dihydrostreptomycin are 0.60 and 1.30, respectively. The organism used was *Mycobacterium avium* A-62.

Fig. 1*b*. The same as Fig. 1*a*, except that the organism used was *M. tuberculosis* strain H37Rv. The slope is 2.10 for both cases.

DISCUSSION

In the present work the antibacterial activity of drugs was determined during the logarithmic phase of growth; the amount of growth of the mycobacteria was observed directly by placing the $\perp$ culture tubes ($\perp$) in the photometer.

Table 1. *The ID 50 doses of various drugs calculated by using the method of Treffers (1956) from the data obtained by measuring the growth of Mycobacterium tuberculosis H37 Rv and M. avium A-62 in rocked 1 tube cultures*

Drug	Test organism			
	Strain A-62		Strain H37Rv	
	ID 50 dose			
	($\mu\text{g./ml.}$)	(M)	($\mu\text{g./ml.}$)	(M)
Dihydrostreptomycin	10	2×10^{-5}	0.01	2×10^{-8}
Viomycin	5	—	0.1	—
Na- <i>p</i> -aminosalicylate	> 500	$> 10^{-3}$	0.2	10^{-6}
Tibion	500	2×10^{-3}	1.0	5×10^{-6}
Pyrazinamide	2	2×10^{-5}	500	5×10^{-3}
Levulinic acid oxime	> 500	3×10^{-3}	100	5×10^{-4}
Isonicotinic hydrazide	5	5×10^{-5}	0.05	4×10^{-7}
Angstromycin	> 500	—	> 500	—
N-Hydroxy-2-pyridine thione	100	10^{-3}	0.02	2×10^{-7}
Pyridomycin	> 500	—	> 500	—

The time needed to determine an ID 50 dose was short, both as regards the time interval between inoculation and observation of growth and the time needed to determine the amount of growth or the % inhibition. It took only 8 hr. with *Mycobacterium avium* and 48 hr. with *M. tuberculosis* strain H37 Rv for reproducible data for antibacterial activity to be obtained.

The slope of the curve, the reproducibility, the determination of drug resistance and other problems were discussed by Treffers (1956) who said 'the drugs acting on the same species of organism, or one drug acting on two species, may, by coincidence, exhibit response curves of identical slope without warranting the conclusion that the mechanisms of action are identical, or even related in any way. A change in slope, with change in the distributions of susceptibilities is at least highly suggestive that some alteration in the mechanism of action has occurred.' The slopes for viomycin and dihydrostreptomycin in Fig. 1*b* (strain H37 Rv) are the same (2.10). The value for streptomycin with *Escherichia coli* was 2.10 (Treffers, 1956). On the other hand, *Mycobacterium avium* strain A-62 showed different values, the slope for viomycin being 0.60 and for dihydrostreptomycin 1.30. At least it can be considered that the site of action of viomycin is different from that of dihydrostreptomycin with *M. avium* A-62.

The present results confirm that the strains used in testing must be carefully selected. Dihydrostreptomycin, sodium *p*-aminosalicylate, Tibion and N-hydroxy-2-pyridine thione showed strong activity against *Mycobacterium tuberculosis* strain H37 Rv, while they did not show any marked activity against *M. avium*.

There are two defects in the present techniques. The optical density is used to determine the growth of organisms, so that it is rather tedious to test a coloured sample in a high concentration. Coloured control should be deducted in each determination. Since Tween 80 is used in a high concentration (1 %),

an unspecific reversal (antagonism) and acceleration (synergism) cannot be denied (Mima, 1953; Youmans & Youmans, 1948). But so far as the drugs used in this experiment are concerned, no such reversal or acceleration was observed. The antagonizing effect of Tween 80 against antibacterial activity of drugs was very similar to that of serum albumin (Natori, 1958).

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