IN-VITRO INHIBITION OF MYCOBACTERIA BY VIRIDANS STREPTOCOCCI

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SUMMARY. Eleven strains of *Mycobacterium tuberculosis* and 13 other species of mycobacteria were examined for susceptibility to 26 strains of viridans streptococci. Susceptibility was detected by the simultaneous antagonism technique, but not by tests of deferred antagonism. It is concluded that in-vitro inhibition of mycobacterial growth by actively growing streptococci is due to a peroxide-mediated antagonism which is too variable to be of value for identification. However, a standard bactericidal test with hydrogen peroxide is worth investigation because this might provide a simple inexpensive aid for the identification of *M. avium-intracellulare* serotypes.

INTRODUCTION

In a previous report (Allen and Swaffield, 1982) *Streptococcus sanguis* II and *S. mitis* were shown to be the main cause of delayed contamination of cultures of *Mycobacterium tuberculosis*. On further examination of a few strains of streptococci, in-vitro antagonism towards *M. tuberculosis* was observed. Other workers have reported streptococcal antagonism to mycobacteria. Gelbert, Tonaki and Adams (1974) isolated, but did not identify, an α -haemolytic streptococcus that inhibited growth of *M. intracellulare*, *M. avium* and other closely related species. Darling and Hart (1976) described the antagonistic action of *S. salivarius* and *S. faecalis* to *M. tuberculosis*, *M. kansasii* and scotochromogenic species, but the two strains they tested did not inhibit growth of *M. intracellulare*, *M. avium* or *M. fortuitum*. It has been suggested (Gelbert *et al.*, 1974; Darling and Hart, 1976) that this apparently selective antagonism might be of value for the in-vitro identification or typing of mycobacteria. The present investigation was designed to identify the nature of streptococcal antagonism and to determine its value in the identification of mycobacteria.

MATERIALS AND METHODS

Bacterial strains. The “producer” strains of streptococci were from a collection of cultures isolated from contaminated Lowenstein–Jensen (LJ) cultures during a previous investigation (Allen and Swaffield, 1982). The 26 strains used were *S. sanguis* II (17 strains) and *S. mitis* (9). Purity checks were made on blood-agar medium and cultures were stored as a suspension at -196°C . Cultures used as indicators of antagonism were 11 strains of *M. tuberculosis* and 13

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TABLE I
Indicator strains of M. tuberculosis

Strain no.	Origin	Virulence for guinea-pig	Phage type	Susceptibility to peroxide*
I2646	Britain	High	A	R
79499	Madras	High	A	R
J6729	Burma	High	A	R
79112	Burma	Low	I	S
79157	Madras	Low	I	S
79500	Madras	Low	I	S
J6720	Teheran	High	B	R
J6198	Britain	High	B	R
J6839	Burma	High	B	R
H37Rv	USA	High	B	R
B1453†	Britain	Low	Not tested	S

* Percentage of cfu surviving exposure to 0.02% H₂O₂ for 90 min at 37°C. Resistant (R) > 10% survivors; sensitive (S) ≤ 10% survivors.

† Isoniazid-resistant (MIC > 50 mg/L in LJ medium) catalase-negative strain.

TABLE II
Indicator strains of mycobacteria other than M. tuberculosis

Species	Origin
<i>M. africanum</i>	Pasteur Institute Paris
<i>M. avium</i>	NCTC8559
<i>M. bovis</i>	Human isolate
<i>M. bovis</i> var BCG	Glaxo strain
<i>M. flavescens</i>	NCTC10271
<i>M. fortuitum</i>	NCTC10394
<i>M. gordonae</i>	NCTC10267
<i>M. intracellulare</i>	NCTC10425
<i>M. kansas</i>	NCTC10268
<i>M. marinum</i>	NCTC2275
<i>M. phlei</i>	NCTC10266
<i>M. scrofulaceum</i>	NCTC10803
<i>M. smegmatis</i>	ATCC607

NCTC: National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT.

strains of other species of mycobacteria. Strains of *M. tuberculosis* with well defined in-vitro and in-vivo characteristics were selected from the culture collection in this department. The main characteristics of these strains are presented in table I. Table II lists the origin of the other strains that were selected to provide a representative sample of species of mycobacteria. All cultures of mycobacteria were stored as a suspension at -196°C.

Culture media. Liquid cultures of streptococci were grown in Todd-Hewitt Broth (THB) (Difco 0492) for 24 h at 37°C. Liquid cultures of mycobacteria were grown in 7H9 Tween-albumin Medium (Difco 0713) at 37°C. Slow-growing mycobacteria were incubated for 14 days and rapid growing species for 4 days.

Solid media for the detection of antagonism were Oleic Acid Albumin Agar (7H11) (Difco 0838) and LJ medium. Plates of 7H11 agar, supplemented with Oleic acid-albumin-dextrose-catalase Complex (Difco 0722), were prepared by pouring 20 ml of molten agar into 9-cm diameter plastic or glass petri dishes. Slopes of LJ medium, with or without benzylpenicillin 60 mg/L, were prepared in 25-ml screw-capped glass containers and solidified by inspissation at 85°C for 60 min.

Simultaneous antagonism. Four wells 8.0 mm in diameter were cut in 7H11-agar plates by means of a sterile metal punch. Plates were inoculated by spreading 0.1 ml of indicator culture over the agar surface with a glass rod. Four drops (about 0.12 ml) of producer cultures in THB were delivered into each well. Plates were sealed in polyethylene bags and incubated at 37°C for 5 days (rapid growers) or 21 days (slow growers). The resultant zones of inhibition were measured with vernier callipers. Tests were also made on LJ medium, with and without benzylpenicillin, with *M. tuberculosis* strains I2646 and B1453 and *M. intracellulare* as indicators. Slopes of media were inoculated with 10 µl of a tween-albumin culture of the mycobacteria. Paper disks, 5 mm in diameter and soaked in a THB culture of streptococci, were placed on the centre of each slope. Cultures were incubated for 3 weeks at 37°C.

Deferred antagonism. Producer strains were inoculated as a streak, approximately 10 mm wide, on duplicate 7H11 agar plates in glass petri dishes. One culture from each pair was incubated at 37°C for 48 h and the other culture at 37°C for 14 days. After incubation, growth was removed with a glass microscope slide and the plate exposed to chloroform vapour for 30 min. To remove residual chloroform, agar surfaces were exposed to a laminar airflow for 30 min. Indicator strains were inoculated at right angles to the producer streak culture with a metal applicator which delivered nine indicator cultures simultaneously. The nine indicator strains were *M. tuberculosis* strains I2646, 79112, J6720, H37Rv and B1453, *M. kansasii*, *M. intracellulare*, *M. bovis* and *M. bovis* var BCG. Plates were incubated until macroscopic growth could be recorded for all strains.

Inhibitory action of culture fractions. Cultures of streptococci in THB, cell-free filtrates, and washed cells were examined for inhibitory effect against *M. tuberculosis* strains I2646 and B1453, *M. kansasii* and *M. intracellulare* by the simultaneous antagonism technique. Overnight THB cultures (10 ml) were used to test complete cultures. Cell-free filtrates were prepared by centrifuging cultures and filtering the supernatant fluid through 0.22-µm pore-size Sterivex® GS filter units (Millipore Corporation). Deposited cells were washed three times in 10-ml volumes of Hanks's Buffered Salt Solution (HBSS) (Gibco Europe 4025) then resuspended in 10 ml of HBSS.

Inactivation of inhibitory effect by catalase was studied by the following techniques: plates of 7H11 agar containing purified thymol-free catalase powder from bovine liver at concentrations of 10, 100 and 1000 Sigma units/ml were used to detect antagonism by streptococci against *M. tuberculosis* (strains I2646 and B1453) and *M. intracellulare* by the simultaneous antagonism technique. The effect of catalase diffusion was studied on 7H11 agar plates in which two ditches, approximately 5 mm × 70 mm, were made with a metal cutter. The ditches were separated by about 8 mm at the closest point and about 40 mm at the most distant point, thus forming an unconnected "V" shape. Plates were surface inoculated with *M. tuberculosis* strains I2646 and B1453 and *M. intracellulare*. One ditch was filled with catalase solution 1000 Sigma units/ml and the other with overnight THB culture of streptococci. By these techniques the three strains of mycobacteria were tested against 26 cultures of streptococci. Plates were incubated as in previous experiments.

The pH within zones of inhibition produced by streptococci on plates seeded with *M. tuberculosis* strain B1453, *M. kansasii*, *M. intracellulare* and *M. bovis* var BCG was measured with a Radiometer PHM63 digital pH meter fitted with a Russel 3-mm stem spear combination electrode.

Detection of peroxide production during growth of streptococci was by the benzidine blood agar (BBA) method (Kraus *et al.*, 1957). Heart Infusion Agar (Difco 044) with lysed horse red cells 0.5% and 3,4,3',4'-tetra-aminobiphenyl hydrochloride (BDH Chemicals) 250 mg/L was poured in 20-ml volumes in 9-cm petri dishes. Plates were surface inoculated with cultures of streptococci and incubated aerobically at 37°C for 4 days. Duplicate tests were incubated in anaerobic conditions.

Susceptibility to hydrogen peroxide. *M. tuberculosis* strains H37Rv and B1453, *M. bovis* var BCG and *M. intracellulare* were tested for peroxide susceptibility by the method of Subbaiah *et al.* (1960). In brief, 0.2 ml of each culture, grown in 7H9 medium, was incubated with 3.8 ml of phosphate buffer at pH 7.0 for 90 min (buffer control), and an additional 0.2 ml was incubated with 3.8 ml of buffer containing 0.02% v/v H₂O₂ (test) for 90 min at 37°C. After incubation, viable counts were set up on samples from the buffer control and the test. The percentage of

organisms surviving exposure was calculated by dividing the number of colony-forming units (cfu) in the suspension exposed to H_2O_2 by the number of cfu in the buffer control, and multiplying by 100.

RESULTS

An example of streptococci inhibiting *M. tuberculosis*, by the simultaneous antagonism test, is illustrated in fig. 1. Zones of inhibition ranged from 13 mm to 40 mm, but the characteristic rough colonial morphology of *M. tuberculosis* often made accurate measurement difficult. As shown in table III, there was no association between inhibition by streptococci and the well defined characteristics of 10 of the 11 strains of *M. tuberculosis* used as indicators. However, strain B1453, an isoniazid-resistant catalase-negative strain, was susceptible to 25 (96%) strains of streptococci. Table IV lists the results obtained when 13 other species of mycobacteria were cultured simultaneously with streptococci. *M. africanum*, *M. avium*, *M. bovis*, *M. fortuitum*, *M. gordonae*, *M. phlei* and *M. scrofulaceum* were resistant to most strains of streptococci. However, *M. intracellulare* was inhibited by all 26 strains of streptococci and *M. bovis*

FIG. 1.—An example of variable streptococcal inhibition of *M. tuberculosis* on oleic-acid albumin agar.

TABLE III

Effect of streptococci on strains of M. tuberculosis by the simultaneous antagonism technique

Strain of <i>M. tuberculosis</i>	Number (and percentage) of 26 strains of streptococci producing zones of inhibition
I2646	13 (50)
79499	5 (19)
J6729	1 (4)
79112	6 (23)
79157	1 (4)
79500	14 (54)
J6720	0
J6198	4 (15)
J6839	2 (8)
H37R _v	6 (23)
B1453	25 (96)

TABLE IV

Inhibitory effect of streptococci on different species of mycobacteria

Species	Number (and percentage) of 26 strains of streptococci producing zones of inhibition
<i>M. africanum</i>	1 (4)
<i>M. avium</i>	0
<i>M. bovis</i>	2 (8)
<i>M. bovis</i> var BCG	23 (88)
<i>M. flavescens</i>	6 (23)
<i>M. fortuitum</i>	0
<i>M. gordonae</i>	1 (4)
<i>M. intracellulare</i>	26 (100)
<i>M. kansasii</i>	9 (35)
<i>M. marinum</i>	5 (19)
<i>M. phlei</i>	2 (8)
<i>M. scrofulaceum</i>	1 (4)
<i>M. smegmatis</i>	10 (38)

TABLE V

Effect of streptococcal culture fractions on four cultures of mycobacteria

Indicator culture (and strain no.)	Number (and percentage) of culture fractions of 26 strains of streptococci producing an inhibition zone in		
	complete medium	culture supernate	washed cells
<i>M. tuberculosis</i> (I 2646)	13 (50)	0	5* (19)
<i>M. tuberculosis</i> (B 1453)	25 (96)	0	19† (73)
<i>M. intracellulare</i>	26 (100)	0	24 (92)
<i>M. kansasii</i>	9 (35)	0	0*

* $p < 0.01$. † $p < 0.05$.

TABLE VI

Effect of catalase on streptococcal inhibition of mycobacteria during simultaneous growth on oleic-acid albumin agar

Indicator culture (and strain no.)	Number (and percentage) of 26 strains of streptococci producing an inhibition zone in the presence of catalase at a concentration (Sigma units/ml) of		
	10	100	1000
<i>M. tuberculosis</i> (12646)	13 (50)	1 (4)*	0
<i>M. tuberculosis</i> (B1453)	25 (96)	22 (85)	1 (4)*
<i>M. intracellulare</i>	26 (100)	22 (85)	1 (4)*

* $p = < 0.001$.

var BCG by 23 (88%). In similar tests on LJ medium, zones of inhibition occurred on drug-free medium but not on medium containing benzylpenicillin. Although the THB inoculum used on paper disks was considerably less (0.01 ml) than that used in wells on 7H11 agar plates (0.12 ml), 5 (19%) strains of streptococci were antagonistic to *M.*

FIG. 2.—Surface growth of *M. tuberculosis* showing protection provided by diffusion of catalase into the medium. Ditch A contained catalase, ditch B contained THB culture of streptococci.

tuberculosis strain I2646, 21 (81%) to strain B1453 and 22 (85%) to *M. intracellulare*. In contrast, the 26 strains of streptococci failed to inhibit any of the nine strains of mycobacteria by the deferred antagonism test.

There was no significant difference between the activity of complete THB culture of streptococci and washed cells against *M. intracellulare*; however, washed cells were significantly less active against *M. kansasii* ($p < 0.01$) and strains I2646 ($p < 0.01$) and B1453 ($p < 0.05$) (table V). Tests on culture filtrates failed to detect antagonism. The effect of catalase on antagonism by streptococci against catalase-positive (I2646) and catalase-negative (B1453) strains and the highly susceptible *M. intracellulare* are presented in table VI. The addition of catalase 100 units/ml significantly reduced the number of streptococci inhibiting the catalase-positive strain I2646, but a concentration of catalase 1000 units/ml was necessary significantly to reduce inhibition of the highly susceptible strains B1453 and *M. intracellulare*.

Tests in which cultures of mycobacteria were exposed to streptococcal inhibition and diffusion of catalase resulted in neutralisation of antagonism at the point where maximum contact occurred (fig. 2).

The pH within zones of inhibition on 7H11 agar plates was not changed sufficiently to account for failure of growth of mycobacteria. Uninoculated agar was found to have a pH 6.6 and the lowest value recorded within zones was pH 5.8. Mycobacteria have been successfully cultured on agar media at pH 5.5 (Stottmeier *et al.*, 1967) and on LJ medium adjusted to pH 5.1 (Tripathy *et al.*, 1970).

On BBA medium, incubated aerobically, all 26 strains of streptococci yielded growth of black colonies indicative of peroxide production. In contrast, when the tests were performed in anaerobic conditions the same strains failed to produce peroxide.

Tests for susceptibility to hydrogen peroxide gave results similar to previous experience in this laboratory with control strain H37Rv (50% survivors) and B1453 (<0.1% survivors). *M. bovis* var BCG (<1% survivors) and *M. intracellulare* (<1% survivors) were highly susceptible to peroxide in these standard test conditions.

DISCUSSION

The phenomenon of in-vitro bacterial antagonism has been recognised for several decades (Aly and Shinefield, 1982). In most instances antagonism is caused by bacteriocins which act only on strains of the same or closely related species. However, Tagg *et al.* (1976) considered the bacteriocins of some gram-positive species to be distinct from those of gram-negative species in that they have a wider species activity. Dajani *et al.* Tom and Law (1976) isolated bacteriocins (termed viridins) from *S. sanguis* and *S. mitis* which were shown to have a broad spectrum of activity including inhibition of several gram-negative species. There have also been several studies in which in-vitro inhibition by α -haemolytic streptococci was shown to be due to production of inhibitory concentrations of hydrogen peroxide. Holmberg and Hallander (1973) and LeBien and Bromel (1975) detected an accumulation of hydrogen peroxide in cultures of *S. sanguis* and *S. mitis*. The simultaneous antagonism technique, used in this investigation, detected in-vitro antagonism by streptococci against several species of mycobacteria. However, tests by the deferred antagonism technique failed to detect bacterial-interference-indicating factors other than bacteriocins as the cause of antagonism. It is also unlikely that alteration of the pH was the cause of streptococcal antagonism.

The evidence presented here showing that streptococcal inhibition of mycobacterial growth can be prevented or reduced by increasing the concentration of catalase in the medium suggests that peroxide is the most likely cause of antagonism. This conclusion is supported by the finding that the most susceptible strain of *M. tuberculosis* (B1453) is catalase negative and highly sensitive to peroxide. The two other strains found to be highly susceptible to streptococcal antagonism (*M. intracellulare* and *M. bovis* var BCG) were also highly susceptible to peroxide.

In strains of *M. tuberculosis*, isoniazid resistance accompanied by loss of catalase activity and increased susceptibility to peroxide is well documented (Middlebrook 1954; Knox *et al.*, 1956). Increased susceptibility to peroxide also occurs in isoniazid-sensitive, catalase-positive, strains isolated in Southern India (Mitchison *et al.*, 1963). However, there is scant information regarding the peroxide susceptibility of other species of mycobacteria. It is interesting that *M. kansasii*, the most active catalase producer of the strains tested, was sensitive to 9 (35%) of the streptococci. This observation supports the findings of Jackett *et al.* (1978) that possession of catalase is only one feature that confers resistance to peroxide. The other factor(s) have not yet been identified.

The finding that washed bacterial cells were less active than complete culture against three of the four strains of mycobacteria tested and that cell-free filtrates were inactive, agrees with the findings of Holmberg and Hallander (1973) who detected peroxide activity in actively growing cultures, but not in bacteria-free filtrates.

It is clear from the results of this investigation that strains with a high degree of susceptibility to peroxide were the most sensitive to streptococcal antagonism. There was, however, no association between antagonism and the degree of peroxide susceptibility among less sensitive strains.

It is of interest that the strains of streptococci examined by Darling and Hart (1976) failed to inhibit growth of *M. avium* and *M. intracellulare* but the strain used by Gelbert *et al.* (1974) was inhibitory to both species. In the present investigation all 26 strains of streptococci inhibited *M. intracellulare* but not *M. avium*. Because more than 20 serotypes of the *M. avium-intracellulare* complex have been identified (Schaefer, 1979) it would be interesting to examine the susceptibility of a more representative sample. However, peroxide production by streptococci is extremely variable (Thomas and Pera, 1983) and it is unlikely that bacterial interference would provide a sufficiently reproducible method for use as an aid to identification. It would therefore be necessary to develop an in-vitro peroxide-susceptibility test similar to that used by Subbaiah *et al.* (1960) to detect differences between serotypes of the *M. avium-intracellulare* complex.

Streptococcal contamination of LJ cultures may lead to inhibition of mycobacterial growth either by changes in toxicity of malachite green (Marshall, 1958) or by an accumulation of peroxide. Marks and Thomas (1958) demonstrated the benefits of adding benzylpenicillin to LJ medium to reduce contamination, and the present investigation supports this recommendation. It is concluded that in-vitro inhibition of mycobacterial growth by streptococci is due to a peroxide-mediated antagonism which serves no useful purpose in the identification or typing of mycobacteria.

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