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SENSITIVITY TO DRUGS OF AUSTRALIAN LEPTOSPIRAL SEROTYPES

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The *in vitro* susceptibilities to antibiotics of stock strains of various Australian leptospiral serotypes were established. *Leptospira pomona* was highly susceptible to penicillin and erythromycin, susceptible to streptomycin and tetracyclines, but resistant to chloramphenicol and novobiocin. In general, the twelve serotypes tested were susceptible to streptomycin, penicillin and tetracycline, but *L. icterohaemorrhagiae, L. canicola* and *L. zanoni* were more resistant than the other serotypes, especially to penicillin. A newly isolated strain of *L. zanoni* was more sensitive to penicillin, streptomycin, and tetracycline than was the stock strain. Against *L. australis* high concentrations both of penicillin and of streptomycin were bactericidal. In lower concentrations penicillin was bacteriostatic. In high concentrations penicillin caused lysis of leptospirae, but streptomycin and the tetracyclines did not. These results do not agree well with those on the chemotherapy of experimental infections.

Information is lacking on the susceptibility *in vitro* to drugs of many of the serotypes of leptospirae occurring in Australia. Previous studies on susceptibility *in vitro* and on chemotherapy in laboratory animals were concerned mainly with *Leptospira icterohaemorrhagiae* and *L. canicola.* These serotypes are important in other countries because of their high incidence and the clinical severity of the disease they cause in man and animals. The relatively high pathogenicity of *L. icterohaemorrhagiae* for guinea-pigs may also have influenced the choice of this serotype for studies of chemotherapy *in vivo.*

Thirteen leptospiral serotypes are known to cause human infection in Australia. Infections both with *L. icterohaemorrhagiae* and with *L. canicola* occur, but numerically they are not important. *L. australis* (*L. australis A*) and *L. zanoni* (*L. australis B*) are the serotypes responsible for the majority of cases of human infection (Derrick, 1957). *L. pomona* is an important cause of animal disease, but *L. icterohaemorrhagiae* infections are not common and *L. canicola* has not, as yet, been isolated from domestic animals.

The response of various Australian leptospiral serotypes to chemotherapeutic agents *in vitro* was therefore investigated.

METHODS

Leptospiral serotypes

Chemotherapeutic tests were performed on twelve serotypes of leptospirae maintained as stock cultures for use in routine serological tests. The serotypes and strains are shown in the following table.

Serotype	*Strain*	*Serotype*	*Strain*
L. icterohaemorrhagiae	Jackson	*L. pomona*	Staines
L. canicola	Berlin	*L. grippotyphosa*	Valbuzzi
L. zanoni	Zanoni	*L. medanensis*	Ives
L. robinsoni	Robinson	*L. kremastos*	Kremastos
L. australis	Ballico	*L. mini*	Szwajizak
L. esposito	Esposito	*L. hyos*	Mackney

A thirteenth Australian serotype, *L. celledoni*, was not included. In addition, a strain of *L. zanoni*, isolated from a rat in North Queensland and designated " Rat 459," was included.

The stock cultures were maintained by weekly subculture in Schüffner's medium containing 10% rabbit serum. Week old cultures containing numerous organisms were used as seed cultures. The newly isolated strain of *L. zanoni* was stored in a sealed bottle until needed, and the tests were performed on the second subculture.

Chemotherapeutic agents

Standard 1:1,000 dilutions (w/v) of these agents were made up freshly in Schüffner's medium for each test. The substances tested were potassium penicillin G (Penicillin P Leo, Andrews), streptomycin sulphate (Glaxo), chloramphenicol (Chloromycetin, Parke Davis), oxytetracycline hydrochloride (Terramycin, Pfizer), chlortetracycline hydrochloride (Aureomycin, Lederle), novobiocin sodium (Albamycin, Upjohn), erythromycin stearate (Erythrocin, Abbott), nitrofurantin (Furadantin, Smith Kline & French Laboratories), chlorhexidine dihydrochloride (Hibitane, I.C.I.), and sulphamethoxypyridazine (Lederkyn, Lederle).

Method of investigation in vitro

Ten-fold dilutions of the standard antibiotic solutions were prepared, and amounts of 1 ml. were dispensed into sterile test tubes. To each tube was added 0.7 ml. of Schüffner's medium, 0.2 ml. of sheep serum and 0.1 ml. of seed culture, giving an antibiotic dilution of 1:2,000 in the first tube, and there were progressive ten-fold dilutions in subsequent tubes.

Cultures were incubated at 30° C and examined with dark-ground illumination at a magnification of ×150 after 4 days and again after 10 days. Control cultures, containing Schüffner's medium in place of antibiotic solution, were included in every test. The test of *L. pomona* with streptomycin was repeated in each series of tests, seven times in all.

The cultures were examined for survival of the seed culture and for multiplication of the surviving organisms. Only motile organisms were recorded as surviving treatment, but the presence of non-motile organisms was recorded. The density of the test cultures was assessed by comparison with control cultures.

Design of experiments in vitro

(*a*) *L. pomona* was incubated in the presence of ten-fold dilutions of the eleven chemotherapeutic agents previously listed, and values for the end-point of growth were obtained.

(*b*) The twelve stock culture serotypes were incubated in the presence of ten-fold dilutions of penicillin, streptomycin, tetracycline, and nitrofurantin, and growth end-point values were obtained.

(*c*) The newly isolated strain of *L. zanoni* was similarly tested against nine chemotherapeutic agents.

(*d*) *L. pomona* was incubated with three-fold dilutions of penicillin, tetracycline, nitrofurantin and chlorhexidine to obtain a more precise estimate of growth end-point values.

(*e*) The tests described above depended on dark-ground examination of the treated cultures. The validity of this procedure was tested by subculturing the treated cultures. Ten-fold dilutions both of penicillin and of streptomycin were inoculated with *L. australis*, the stock culture possessing greatest motility. After 2, 4, 24, 48, 72 and 96 hr, microscopic examinations were

made. At the same time a small quantity of each dilution was subcultured into sufficient medium to achieve a 100-fold dilution of the antibiotic carried over. Subcultures were examined at weekly intervals for 4 weeks.

RESULTS

(*a*) Table 1 lists the end-points of growth when *L. pomona* was incubated in the presence of each of eleven chemotherapeutic agents. A determination of the end-point for *L. pomona* with streptomycin was performed in each experiment. In all seven tests the 2×10^5 dilution completely suppressed growth, but the density of growth in the next dilution varied from test to test. Both erythromycin and penicillin were active in very high dilutions.

TABLE 1

HIGHEST DILUTIONS OF ANTIBIOTICS GIVING COMPLETE INHIBITION OF GROWTH OF *L. POMONA* (STRAIN STAINES) *IN VITRO* AFTER 4 DAYS' INCUBATION

Dilutions are expressed as reciprocals of antibiotic concentrations (in g/ml.). Antibiotics were used in serial ten-fold dilutions

Substance	Dilution	Substance	Dilution
Erythromycin	2×10^{11}	Oxytetracycline	2×10^5
Penicillin	2×10^8	Nitrofurantin	2×10^5
Streptomycin	2×10^5	Chlorhexidine	2×10^5
Tetracycline	2×10^5	Novobiocin	2×10^5
Chlortetracycline	2×10^5	Chloramphenicol	$<2 \times 10^3$
		Sulphamethoxypyridazine	$<2 \times 10^3$

Penicillin was the only substance with which one dilution completely suppressed growth, and the next dilution allowed the same density of growth as in control tubes. With the other antibiotics, one or two tubes showing partial growth separated the dilutions that completely suppressed and those that allowed complete growth. When the tubes showing partial inhibition were re-examined 6 days later, those in which only slight growth had occurred were often negative, but density in the other tubes equalled that in the control tubes.

When examined at 4 days, tubes containing higher concentrations of streptomycin or the tetracyclines contained small numbers of intact non-motile organisms. These degenerate forms had always disappeared by 10 days, and they were not observed in penicillin-treated cultures at either examination.

(*b*) The end-points of growth when the twelve stock cultures were incubated in the presence of four chemotherapeutic agents are shown in Table 2. Again, non-motile organisms were observed in higher concentrations of streptomycin and tetracycline, but not with penicillin. Penicillin gave less clear-cut end-points against *L. icterohaemorrhagiae, L. canicola* and *L. zanoni,* the most resistant serotypes.

(*c*) *L. zanoni,* strain " Rat 459," was incubated with nine chemotherapeutic agents. An end-point value was not obtained for erythromycin, but a dilution of 2×10^7 produced complete inhibition. The dilutions of the other antibiotics producing complete inhibition are given in Table 3.

TABLE 2

HIGHEST DILUTIONS OF ANTIBIOTICS PRODUCING COMPLETE INHIBITION OF GROWTH AFTER 4 DAYS' INCUBATION

Dilutions are expressed as reciprocals of antibiotic concentrations (in g/ml.)

Serotype	Chemotherapeutic agent			
	Streptomycin	Penicillin	Tetracycline	Nitrofurantin
L. icterohaemorrhagiae	2×10^4	2×10^3	2×10^4	$<2\times10^3$
L. canicola	2×10^3	2×10^3	2×10^4	2×10^3
L. zanoni	2×10^4	2×10^4	2×10^4	2×10^4
L. robinsoni	2×10^6	2×10^7	2×10^5	2×10^3
L. australis	2×10^5	2×10^7	2×10^5	$<2\times10^3$
L. esposito	2×10^4	2×10^7	2×10^5	2×10^4
L. pomona	2×10^5	2×10^8	2×10^5	$<2\times10^3$
L. grippotyphosa	2×10^5	2×10^7	2×10^5	2×10^3
L. medanensis	2×10^5	2×10^7	2×10^5	2×10^3
L. kremastes	2×10^5	2×10^7	2×10^6	2×10^3
L. mini	2×10^5	2×10^7	2×10^6	$<2\times10^3$
L. hyos	2×10^5	2×10^8	2×10^7	2×10^3

TABLE 3

HIGHEST DILUTIONS OF ANTIBIOTICS PRODUCING COMPLETE INHIBITION OF GROWTH OF *L. ZANONI* (STRAIN "RAT 459") AFTER 4 DAYS' INCUBATION

Dilutions are expressed as reciprocals of antibiotic concentrations (in g/ml.)

Substance	Dilution	Substance	Dilution
Penicillin	2×10^6	Tetracycline	2×10^5
Streptomycin	2×10^5	Chloramphenicol	2×10^3
Oxytetracycline	2×10^5	Nitrofurantin	2×10^3
Chlortetracycline	2×10^5	Chlorhexidine	$<2\times10^3$

TABLE 4

COMPARISON OF RESULTS OBTAINED BY DARK-GROUND EXAMINATION AND BY CULTURE WITH *L. AUSTRALIS* AND PENICILLIN

Top line: +, motile organisms on microscopic examination; −, no motile organisms on microscopic examination. Bottom line: +, growth on subculture; −, no growth on subculture

Time (hr)	Reciprocal of dilution of penicillin								
	2×10^3	2×10^4	2×10^5	2×10^6	2×10^7	2×10^8	2×10^9	2×10^{10}	Control
2	+	+	+	+	+	+	+	+	+
	−	−	−	−	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+
	−	−	−	−	+	+	+	+	+
24	+	+	+	+	+	+	+	+	+
	−	−	−	−	+	+	+	+	+
48	−	−	−	−	−	+	+	+	+
	−	−	−	−	−	+	+	+	+
72	−	−	−	−	−	+	+	+	+
	−	−	−	−	−	+	+	+	+
96	−	−	−	−	−	+	+	+	+
	−	−	−	−	−	+	+	+	+

(*d*) Three-fold dilutions of several agents were incubated with *L. pomona*. A dilution of 2×10^4 of nitrofurantin, 1.8×10^5 of chlorhexidine, 5.4×10^5 of streptomycin and 4.86×10^6 of tetracycline produced complete inhibition.

(*e*) The results of microscopic and cultural examinations of cultures of *L. australis* incubated with penicillin (Table 4) and streptomycin (Table 5) are compared. At

TABLE 5

COMPARISON OF RESULTS OBTAINED BY DARK-GROUND EXAMINATION AND BY CULTURE WITH *L. AUSTRALIS* AND STREPTOMYCIN

Top line: +, motile organisms on microscopic examination; −, no motile organisms on microscopic examination. Bottom line: +, growth on subculture; −, no growth on subculture

Time (hr)	Reciprocal of dilution of streptomycin								
	2×10^3	2×10^4	2×10^5	2×10^6	2×10^7	2×10^8	2×10^9	2×10^{10}	Control
2	+	+	+	+	+	+	+	+	+
	−	−	−	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+
	−	−	−	+	+	+	+	+	+
24	+	+	+	+	+	+	+	+	+
	−	−	−	+	+	+	+	+	+
48	−	−	−	+	+	+	+	+	+
	−	−	−	−	+	+	+	+	+
72	−	−	−	+	+	+	+	+	+
	−	−	−	−	+	+	+	+	+
96	−	−	−	+	+	+	+	+	+
	−	−	−	−	+	+	+	+	+

2 and 4 hr no microscopic changes were observed with either antibiotic, but cultures from tubes containing the highest concentrations were negative. At 24 hr motile leptospirae were detected in all tubes, although only small numbers were present in the higher concentrations and subcultures of these showed no growth. At 48 hr, no organisms could be detected microscopically in the higher concentrations. The organisms detected in all the penicillin dilutions were viable when subcultured, but those in the first streptomycin tube showing growth were not. Thus the 2×10^6 dilution of penicillin showed bactericidal action by 2 hr, and the 2×10^7 dilution after 48 hr. For streptomycin, the 2×10^5 dilution was bactericidal at 2 hr, and the 2×10^6 at 48 hr.

DISCUSSION

Numerous studies on the chemotherapeutic responses of leptospirae grown in fluid media have been published, and large differences in sensitivity between and within serotypes have been observed. Comparison of these reports is difficult because of differences in experimental methods. Results have been based on different criteria including suppression of growth and failure to demonstrate viable organisms on subculture, as in the present study, loss of motility (Goldberg & Logue, 1956)

TABLE 6

ANTIBIOTICS ARRANGED IN DESCENDING ORDERS OF ACTIVITY AGAINST *L. ZANONI IN VITRO* AND *IN VIVO*

Reciprocal of dilution causing inhibition *in vitro*		Effective single dose (mg) against acute infection in mice	
(1) Erythromycin	2×10^7	(1) Chlortetracycline	<2
(2) Penicillin	2×10^6	Tetracycline	<2
(3) Streptomycin	2×10^5	(2) Oxytetracycline	4
(3) Oxytetracycline	2×10^5	(3) Streptomycin	8
(3) Chlortetracycline	2×10^5	Erythromycin	8
(3) Tetracycline	2×10^5	(4) Penicillin	>8
(4) Chloramphenicol	2×10^3	(5) Chloramphenicol	>8

and inhibition of respiration (Fulton & Spooner, 1956). Hoag & Bell (1955) showed that results varied with different media, and Katsura & Yoshida (1957) reported that the stage of growth of the culture was also important.

Antibiotic activity *in vitro* may not be closely correlated with activity *in vivo*. In Table 6, the antibiotics are arranged in order of effectiveness *in vitro* against a recently isolated strain of *L. zanoni* (present study), and against acute leptospirosis in mice produced by another strain of *L. zanoni* (Spradbrow, 1963). Not only are the magnitudes of the differences between antibiotics reduced, but the antibiotics are arranged in different orders in the two groups. Erythromycin and penicillin, the most effective antibiotics *in vitro*, had only low activity in the experimental animal. The low activity of chloramphenicol *in vitro* was correlated with its lack of action *in vivo*.

Van Thiel (1957) investigated the susceptibility of strains of *L. icterohaemorrhagiae* to antibiotics *in vivo* and *in vitro*. He noted discrepancies between the results of *in vitro* and *in vivo* tests, and referred particularly to the high effectiveness of streptomycin in experimental animals, and to its poor activity *in vitro*.

Of the eleven substances tested against *L. pomona*, erythromycin and penicillin are of special interest, because of the extremely high dilutions at which they were active. Ormsbee (1953) noted that erythromycin was 8 to 40 times more effective, on an equimolar basis, than penicillin in the treatment of *L. icterohaemorrhagiae* infections in the chick embryo. Chloramphenicol and sulphamethoxypyridazine were without action.

In Table 2, the serotypes are arranged in order of serological relationships. This is also roughly the order of increasing susceptibility to penicillin and tetracycline. The variation between serotypes in sensitivity to penicillin was very great. By contrast, the dilutions at which streptomycin was effective were more uniform. In general, *L. icterohaemorrhagiae*, *L. canicola* and *L. zanoni* were more resistant to antibiotics, especially to penicillin, than were the other serotypes. The relatively high resistances of *L. icterohaemorrhagiae* and of *L. canicola* to penicillin may explain the poor responses obtained in Britain with this drug in the treatment of human leptospirosis due to these serotypes (Broom, 1951). In Australia, where infection with the more sensitive serotypes predominates, penicillin is the favoured antibiotic (Doherty, 1956).

The newly isolated strain of *L. zanoni* was more sensitive than the stock culture to penicillin, streptomycin and tetracycline. Führer (1952) also noted that laboratory cultures were more resistant than recently isolated strains to antibiotics.

Hoag & Bell (1955) observed that appearance of leptospirae on dark-ground examination was of no value for determining the ability of the organism to grow on subculture. In the present study, subcultures from tubes which contained no motile organisms always remained sterile; but subcultures containing motile organisms did not always produce growth. At 2, 4 and 24 hr the highest concentrations of both antibiotics contained motile organisms, but these were not capable of initiating growth.

At 2, 3 and 4 days, the 2×10^6 dilution of streptomycin was the highest concentration containing motile organisms, but these were not viable on subculture. Motile

organisms in the highest penicillin concentration showing growth, 2×10^7, were viable. The action of penicillin in this concentration was in part bacteriostatic, and that of streptomycin in a concentration that prevented growth was bactericidal. The growth end-point for streptomycin occurred at a higher dilution when determined by subculture than when based on microscopical examination. For penicillin, both methods gave the same end-point.

High concentrations of streptomycin and the tetracyclines often contained non-motile, degenerating leptospirae, but high concentrations of penicillin were free of organisms. Penicillin, in these high concentrations, appeared to have lytic action on leptospirae. Babudieri (1949) examined leptospirae exposed to bactericidal concentrations of penicillin under the electron microscope, and observed rapid and almost complete lysis of the organisms. Mackay-Dick & Robinson (1959) attributed the Jarisch-Herxheimer reaction that follows penicillin therapy of human leptospirosis to lysis of the organisms and release of toxins. If penicillin has this action *in vivo*, the antibody response following penicillin treatment may differ from that produced by an antibiotic that does not disrupt the leptospirae.

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