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CHEMOTHERAPY OF EXPERIMENTAL LEPTOSPIRAL INFECTION IN MICE

BY

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A strain of *Leptospira zanoni* was used to produce chronic renal infections in young white mice. A variant of this strain produced an acute disease with over 50% mortality. The responses of both forms of disease to chemotherapy were studied. When treatment of the acute disease was initiated before jaundice occurred, suitable single doses of streptomycin, chlortetracycline, tetracycline, erythromycin, oxytetracycline and oxytetracycline (in oil) prevented death and chronic renal infection in a high percentage of mice. Bicillin, a long-acting penicillin preparation, was more effective than other penicillins, but it prevented the development of chronic renal infection in only half the treated mice. Streptomycin was the only antibiotic of which a single administration regularly cured the chronic renal infections: chlortetracycline, tetracycline and oxytetracycline (in oil) were partially effective. Oxytetracycline, chloramphenicol, Bicillin, fortified penicillin, procaine penicillin and potassium penicillin had no permanent action. The suitability of mice as laboratory animals in the study of experimental leptospirosis and the need for complete cure of carriers of chronic renal infection are emphasized. The above findings indicate that streptomycin is the drug of choice for the treatment of leptospirosis in animals, and that it is worthy of further trial in man.

Guinea-pigs and golden hamsters have been the animals most commonly used in laboratory studies on leptospiral infections. Golden hamsters are not available in Australia, and both species are less convenient to handle than mice, which are recognized as the ideal laboratory animal. The possibility of using mice in the study of various aspects of experimental leptospirosis was, therefore, investigated.

Larson (1941) reported that young white mice were extremely susceptible to *Leptospira icterohaemorrhagiae* infection, but Neghme, Christen, Jarpa & Agosin (1951) found that different strains of mice varied greatly in susceptibility to this organism. Olejnik & Shneyerson (1952) established "*L. geffeni*" infection in mice and studied the action of oxytetracycline on this infection. Emanuel (1959) reported that mice were susceptible to many of the leptospiral serotypes isolated in North Queensland. *L. australis* (*L. australis A*) and *L. grippotyphosa* produced severe infections, *L. icterohaemorrhagiae*, *L. canicola*, *L. zanoni* (*L. australis B*), *L. hyos*, *L. esposito* and strain "Robinson" produced moderate to mild infections. Survivors of all grades of infection became permanent renal carriers.

Strains of *L. zanoni* were obtained and used for chemotherapeutic studies in mice.

METHODS

Strains of L. zanoni. Attempts were made to infect mice with four strains. Strain "Galanopoulas" of human origin produced no clinical evidence of disease, but mice became chronic urinary carriers. Leptospirae became detectable in the urine 8 to 10 days after inoculation and were excreted in large numbers, and organisms were still detectable in the urine after 12 months. After only one mouse passage the virulence of this strain was greatly increased, and the strain produced severe icteric infections with 50 to 90% mortality in newly weaned mice. All surviving mice became chronic urinary carriers. All chemotherapeutic tests recorded in this paper were carried out against infections with this strain.

A strain of *L. zanoni* isolated from a wild mouse in North Queensland produced a disease similar to the chronic form described above. Two strains similarly isolated from rats produced only transient renal infections. Organisms were excreted in very small numbers, and were detectable for only a few days.

Mice. Newly weaned mice of 15 to 20 g were used. To facilitate collection of urine, female mice were used whenever possible. In one experiment in which male mice were included, both treated and control groups contained equal numbers of male and female mice.

Inoculum. Well-grown 7 to 14 day cultures of *L. zanoni*, strain "Galanopoulas," in Schüffner's medium containing 20% rabbit serum, were used for inoculating mice. The parent strain, which produced urinary carriers, was maintained by occasional passage through Schüffner's medium. Virulence of the highly pathogenic variant was maintained by mouse passage, and by the seeding of cultures with blood from the heart of a jaundiced moribund mouse.

Mice were infected intraperitoneally with 0.2 ml. of culture.

Examination of urine. The urine of each mouse was examined microscopically with dark-ground illumination, before inoculation. Urine was collected by gentle manipulation of the bladder through the abdominal wall. A drop of urine was collected onto a 13 mm diameter circular coverslip which was then inverted onto a clean, new, microscope slide. Six coverslips were placed on each slide, and the preparation was examined at a magnification of $\times 600$.

Leptospirae in the urine of infected mice were easily recognized. Most of the organisms were non-motile, a few showed slow movement about the long axis, and only rarely was urine encountered in which the organisms showed normal movement of translation. The usual smooth hooks at either end, as seen on organisms in culture, were replaced by angular processes. These changes were attributed to the acidity of mouse urine, which was *p*H 5.8 to 6.1 (estimated by indicator papers).

Kidney cultures. Many mice were autopsied, and kidney cultures were made to verify the results of urine examinations. Two small pieces of cortex were removed with sterile instruments and duplicate cultures were made by placing the pieces in Schüffner's medium with 20% rabbit serum. Cultures were incubated at 30° C and examined at weekly intervals for 4 weeks.

Chemotherapeutic agents. Drugs were administered subcutaneously in single doses, which were usually contained in 0.4 ml. of distilled water. Antibiotics used were streptomycin sulphate (Glaxo), oxytetracycline hydrochloride (Terramycin, Pfizer), chlortetracycline hydrochloride (Aureomycin, Lederle), tetracycline hydrochloride (Achromycin, Lederle), chloramphenicol (Chloromycetin, Parke Davis), erythromycin stearate (Erythrocin, Abbott), novobiocin sodium (Albamycin, Upjohn), a long acting penicillin preparation containing benzathine penicillin G, procaine penicillin G and potassium penicillin G (Bicillin, Wyeth), a fortified penicillin preparation containing procaine penicillin and sodium penicillin (Aquacaine C Fortified, C.S.L.), procaine penicillin G (Aquacillin, Faulding), potassium penicillin G (Penicillin P Leo, Andrews) and a preparation of oxytetracycline hydrochloride in mineral oil (Terramycin suspension in oil, Pfizer). Smaller numbers of mice were also tested with chlorhexidine dihydrochloride (Hibitane, I.C.I.), nitrofurantin (Furadantin, Smith Kline & French Laboratories) and hexamine.

Chronic disease. Treatment was not initiated until at least two positive urine samples had been collected. The course of infection after treatment was followed by making repeated dark-ground examinations of the urine. A sample was considered negative if no organisms

were detected in fifty microscope fields. Severity of infection could be roughly graded by estimating the number of organisms per field.

Acute disease. Mice were examined daily for signs of clinical illness. When jaundice occurred it was always very intense, and very few jaundiced mice survived. Death occurred 1 to 2 days after mice became jaundiced. An occasional mouse died without jaundice.

Autopsy revealed intense yellowing of subcutaneous tissue and large subcutaneous areas of haemorrhage. The lungs were very pale, with a few scattered haemorrhages of 1 to 2 mm diameter. The liver was enlarged, soft and yellow, and the kidneys were yellow.

Leptospirae could be recovered by culture of heart-blood, liver, kidney or spleen of freshly dead mice.

Surviving mice were examined for chronic renal infection, by dark-ground microscopic examination of urine, and by culture of kidney tissue.

RESULTS

Correlation of findings with dark-ground examination of urine and with culture of kidneys

Duplicate cultures of kidney tissues were made from fifty-four treated mice which were considered to be free of infection on the basis of negative findings with repeated urine examinations. No growth of leptospirae occurred during 4 weeks' incubation. Kidneys were also cultured from twenty-six mice with detectable leptospiruria. Only twenty yielded cultures of leptospirae.

Treatment of chronic infection

The results of chemotherapy of established chronic urinary carriers are shown in Table 1. Streptomycin, Bicillin, chlortetracycline, tetracycline and oxytetracycline

TABLE 1

CHEMOTHERAPY OF CHRONIC *LEPTOSPIRA ZANONI* INFECTION IN MICE

All drugs were administered subcutaneously in single doses except where otherwise stated

Substance	Dose (per mouse)	Number of mice			
		Tested	Urine cleared	Relapsed	Permanently cured
Streptomycin	8 mg	48	48	0	48
	4 mg	14	14	3	11
	2 mg	14	14	5	9
	1 mg	8	3	3	0
Bicillin	9,600 units	17	17	17	0
Fortified penicillin	9,600 units	10	1	1	0
Procaine penicillin	9,600 units	10	0		0
Potassium penicillin	9,600 units	10	0		0
Tetracycline	8 mg	23	18	12	6
Chlortetracycline	8 mg	8	8	3	5
	4 mg	9	8	2	6
	2 mg	9	7	4	3
Oxytetracycline	8 mg	7	0		0
Oxytetracycline in oil	8 mg	13	11	2	9
	4 mg	10	9	0	9
	2 mg	10	3	0	3
Erythromycin	1 mg	13	7	7	0
Chloramphenicol	8 mg	8	0		0
Chlorhexidine	4 mg (oral)	4	0		0
Hexamine	4 mg (oral)	4	2	1	1
Nitrofurantin	4 mg (oral)	4	4	2	2
	8 mg×3 days	6	3	3	0
	4 mg×3 days	6	0		0
Untreated control	—	85	0		0
Un-inoculated control	—	85	All urine examinations negative		

in oil, each cleared the urine, as shown by negative urine samples after treatment. However, with most drugs, organisms were again detected at later examinations, and the mice became permanent carriers.

A single dose of 8 mg of streptomycin was, however, highly effective. The urine was usually free of organisms 24 hr after treatment and no relapses occurred, even in mice observed for 164 days after treatment, and all kidney cultures were negative. Relapses sometimes occurred with single doses of 4 and 2 mg, and always with 1 mg. After 5 to 7 days small numbers of organisms appeared in the urine, but even after 40 days the numbers had not returned to those seen in the carrier state before treatment.

The 8 mg dose of Bicillin (9,600 units) produced no permanent cures and was about equivalent in action to 1 mg of streptomycin. The urine was cleared of organisms in 1 to 2 days, but leptospirae were detected again 1 to 5 days later.

Neither procaine nor potassium penicillins (9,600 units) had any action. In preliminary experiments with small numbers of mice, neither chlorhexidine, nitrofurantin nor hexamine appeared to have any significant action.

Chlortetracycline cleared the urine, but relapses occurred after long periods of apparent cure. In one test occasional organisms became detectable in the urine 10 days after it had been cleared, and the excretion rate gradually increased.

The oxytetracycline in oil was almost as effective as streptomycin, but surprisingly oxytetracycline in aqueous solution had no action. Tetracycline and chloramphenicol (8 mg) and erythromycin (1 mg) were also inactive.

Mice inoculated with the strain causing chronic renal infection were also treated during the incubation period.

Table 2 shows the results of various antibiotic treatments at 1 day after inoculation. Mice treated with streptomycin, chlortetracycline, erythromycin or oxytetracycline

TABLE 2

RESULTS OF TREATMENT OF MICE AT 1 DAY AFTER INOCULATION

Mice were inoculated with *Leptospira zanoni* strain, producing chronic infection

Antibiotic	Dose	Number of mice	
		Treated	Carriers
Streptomycin	8 mg	11	0
Chlortetracycline	8 mg	23	0
Erythromycin	8 mg	10	0
Oxytetracycline in oil	4 mg	10	0
Bicillin	9,600 units	6	6
Procaine penicillin	9,600 units	6	6
Novobiocin	8 mg	5	5
Untreated control	—	11	11
Un-inoculated control	—	11	0

in oil did not subsequently become urinary carriers; but renal infection developed in untreated control mice and in mice treated with Bicillin, procaine penicillin and novobiocin.

Acute diseases

Preliminary experiments indicated that chemotherapy with penicillin or streptomycin was not effective, unless initiated before the occurrence of jaundice and clinical

illness. The results of treatment on the first, second or third days after inoculation are summarized in Table 3.

Chlortetracycline, tetracycline and the higher doses of streptomycin, erythromycin and oxytetracycline in oil prevented both death and chronic renal infection. The

TABLE 3

TREATMENT OF ACUTE *L. ZANONI* INFECTION OF MICE AT 1 TO 3 DAYS AFTER INOCULATION

* Probably due to toxicity of oxytetracycline

Substance	Dose (per mouse)	Number of mice			
		Tested	Died	Carrier	Total affected
Streptomycin	8 mg	44	1	2	3
	4 mg	10	2	4	6
	2 mg	10	0	9	9
Potassium penicillin	9,600 units	15	0	14	14
	2,400 units	10	0	10	10
Procaine penicillin	9,600 units	15	1	14	15
	2,400 units	10	1	9	10
Bicillin	9,600 units	30	0	15	15
	2,400 units	10	0	9	9
Fortified penicillin	9,600 units	10	0	10	10
	2,400 units	10	1	9	10
Oxytetracycline	8 mg	15	7*	0	7*
	4 mg	15	0	0	0
	2 mg	15	0	5	5
Oxytetracycline in oil	8 mg	10	0	1	1
	4 mg	10	0	0	0
	2 mg	10	0	4	4
Chlortetracycline	8 mg	15	0	0	0
	4 mg	10	0	0	0
	2 mg	10	0	0	0
Tetracycline	8 mg	11	0	0	0
	4 mg	10	0	0	0
	2 mg	10	0	0	0
Erythromycin	8 mg	10	0	0	0
	1 mg	11	0	11	11
Novobiocin	8 mg	5	5	0	5
Chloramphenicol	8 mg	11	6	5	11
Untreated control	—	87	48	39	87
Un-inoculated control	—	50	0	0	0

four penicillin preparations prevented death in inoculated mice, but Bicillin was the only penicillin preparation that protected some of the mice against chronic infection.

Half the mice treated with 8 mg of oxytetracycline died, but the survivors did not become carriers. A dose of 4 mg protected completely. The deaths with the higher dose may have been due to a combination of infection and of toxicity of oxytetracycline. No deaths occurred in ten uninfected mice nor in seven carrier mice, each treated with 8 mg of oxytetracycline.

Novobiocin and chloramphenicol were ineffective.

Neither streptomycin (8 mg) nor Bicillin (9,600 units) had prophylactic action in groups of five mice when given 24 hr or longer before inoculation with the virulent strain.

DISCUSSION

It is surprising that mice have been so little used in the study of experimental leptospirosis. The present study and that of Olejnik & Shneyerson (1952) indicate that they are very suitable for studies on the chemotherapy of leptospirosis. The mice in these experiments were free from natural leptospiral infections, and *L. zanoni* did not spread from mouse to mouse under cage conditions. Normal mice kept for 3 months in the same cage as heavy carriers did not develop antibodies and did not become urinary carriers.

No practicable laboratory technique appears to be entirely suitable for detecting small numbers of leptospirae in animal tissue. The use of repeated dark-ground microscopic examinations of urine was satisfactory for detecting chronic renal infections in mice. The negative results recorded with treated mice that later " relapsed " were probably due to temporary reduction of the excretion rate to below detectable levels. Culture of kidney tissue isolated leptospirae from only about 80% of known carriers.

Table 4 shows the relative efficiencies of the various antibiotics in the acute and the chronic diseases. During the leptospiraemic stage *L. zanoni* was susceptible

TABLE 4

COMPARATIVE EFFICIENCY OF ANTIBIOTICS IN THE TREATMENT OF ACUTE AND CHRONIC *LEPTOSPIRA ZANONI* INFECTIONS IN MICE

Action	(a) Acute disease	(b) Chronic disease
Effective: (a) Prevented death and occurrence of renal infection in most animals (b) Produced permanent clearing of urine in most animals	Streptomycin, 8 mg Oxytetracycline, 4 mg Oxytetracycline in oil, 4 mg Chlortetracycline, 2 mg Tetracycline, 2 mg Erythromycin, 8 mg	Streptomycin, 8 mg Oxytetracycline in oil, 4 mg
Partially effective: (a) Prevented death, but renal infection occurred (b) Cleared urine of most animals, but some or all relapsed	Streptomycin, 2 mg Bicillin, 2,400 units Fortified penicillin, 2,400 units Procaine penicillin, 2,400 units Potassium penicillin, 2,400 units Oxytetracycline, 2 mg Erythromycin, 1 mg	Streptomycin, 2 mg Bicillin, 9,600 units Chlortetracycline, 2 mg Tetracycline, 8 mg Erythromycin, 8 mg Oxytetracycline in oil, 2 mg
Not effective: (a) and (b) No differences between treated and control groups	Novobiocin, 8 mg Chloramphenicol, 8 mg	Streptomycin, 1 mg Fortified penicillin, 9,600 units Procaine penicillin, 9,600 units Potassium penicillin, 9,600 units Oxytetracycline, 8 mg Chloramphenicol, 8 mg

to a wide variety of chemotherapeutic agents, but only streptomycin and oxytetracycline in oil were fully effective against organisms localized in the kidney tubules. The superiority of Bicillin to other penicillin preparations, and of oxytetracycline in oil to oxytetracycline in aqueous solution indicates that the duration of blood and urine levels of antibiotics is important. Other antibiotics, especially the tetracyclines, might have been more effective if multiple doses had been used.

These observations are not entirely in accord with findings on the sensitivity of leptospirae to antibiotics *in vitro* (Spradbrow, 1963). In general, these organisms

were susceptible to very low concentrations of erythromycin and of penicillin, moderate concentrations of the tetracyclines and of streptomycin, but only to high concentrations of novobiocin, and they showed complete resistance to chloramphenicol. Similar differences between *in vitro* and *in vivo* activity of the sulphonamides were reported by Francis (1952).

Antibiotics have been evaluated with other laboratory animals, with domestic animals, and with man. The value of streptomycin in preventing or curing renal infection in guinea-pigs has been demonstrated with *L. icterohaemorrhagiae* infection (van Thiel, 1957), in hamsters and dogs with *L. canicola* infection (Brunner & Meyer, 1949), and in calves with *L. pomona* infection (Spradbrow, unpublished). In veterinary practice, elimination of the urinary shedder is fundamental to the control of animal leptospirosis, and streptomycin would appear to be the drug of choice.

Doherty (1955, 1956) reported the treatment of a large number of cases of human leptospirosis in North Queensland, and concluded that penicillin had definite therapeutic action, especially if given in high doses. Broad spectrum antibiotics were less effective. The value of penicillin in the treatment of human leptospirosis in other areas is controversial (Fairburn & Semple, 1956 ; Mackay-Dick & Robinson, 1957, 1959 ; Semple, 1959).

There appear to have been no large scale trials with streptomycin in the treatment of human leptospirosis. Van Thiel (1957) suggested that this antibiotic merited further investigation, on the basis of his results with streptomycin in the treatment of *L. icterohaemorrhagiae* infections in guinea-pigs. His suggestion is supported by the results with *L. zanoni* infections in mice in the present study.

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