DOXYCYCLINE PROPHYLAXIS FOR EXPERIMENTAL LEPTOSPIRA INFECTION IN NON-HUMAN PRIMATES AND HAMSTERS

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INTRODUCTION

Leptospirosis is an endemic disease in Thailand that occurs primarily during the rainy season (Bunnag *et al.*, 1983). Although a variety of serovars infect man in Thailand, *Leptospira interrogans* serovar *bataviae (L. bataviae)* is a frequent cause of clinical infection and in Bangkok it is the most common isolate from confirmed cases of human leptospirosis (Charoonruangrit and Boonpacknavig, 1964; Sitprija *et al.*, 1980; Bunnag *et al.*, 1983). Doxycycline has been shown to prevent clinical leptospirosis in soldiers training in Panama (Takafugi *et al.*, 1984); treatment early in the course of infection significantly reduces the duration of illness and prevents the development of urinary tract infection with leptospires (McClain *et al.*, 1984). The purposes of this study were to test the efficacy of doxycycline prophylaxis in non-human primates and hamsters infected with *L. bataviae*, and to determine the sensitivity of local isolates of this serovar to doxycycline *in vitro*.

MATERIALS AND METHODS

The eight *Leptospira interrogans* serovar *bataviae* isolates used in this study were cultured from the blood of febrile patients with clinical leptospirosis. One to two drops of blood were added to 5.0 ml of protein supplemented semisolid Ellinghausen-McCullough-Johnson-Harris(EMJH) media and incubated at 30°C (Turner, 1970). This media was used for all isolation attempts from blood, urine and cerebrospinal fluid (CSF). *In vitro* sensitivity testing was done in liquid EMJH media. Cultures were examined weekly by dark field microscopy and after six weeks, samples without organisms were considered negative.

In the first experiment, 10^8 organisms of an isolate of *L. bataviae* (LC0475) were injected intraperitoneally (IP) into eight healthy, adult, male rhesus monkeys (6-10 kg), negative for *Leptospira* antibody (22 serovars) by microagglutination test (Turner, 1968). Four of the eight were given an oral doxycycline suspension (4-5 mg/kg) in water (1 mg/cc) by a nasogastric tube and the other four were given water alone (diluent). The dose of doxycycline (Vibramycin, Pfizer Laboratories), based on body surface area (Freireich *et al.*, 1966), was equivalent in mg/m^2 to a human dose 100 mg/60 kg. Treatment was given daily beginning the day before injection and continued nine days post injection. In the second experiment, four monkeys received an oral dose (8-10 mg/kg) of doxycycline suspension and four others were given water 2 hours prior to IP injection of 10^7 organisms (LC0475) and again on day 7. Blood, CSF, and urine were cultured by adding one to two drops to separate tubes containing EMJH media. Monkeys were anesthetized with ketamine hydrochloride (10 mg/kg) and the skin over the femoral vein, lumbar spine, and pubis was disinfected prior to bleeding, spinal tap or cystocentesis. If doxycycline treatment coincided with a day of sampling, samples were taken before treatment.

Syrian hamsters (40-60g), 10 groups with 6 animals per group, were injected IP with 1×10^5 organisms (LC0475) in 0.1 ml media or with 0.1 ml of media only. An initial dose of doxycycline (28.8 mg/kg) based on body surface area equivalent to a 200 mg/60 kg dose for man was given by gavage and subsequent doses (14.4 mg/kg) were given daily as shown for each group. Time of death was recorded for each group and all surviving hamsters were killed at 6 weeks with CO_2. Kidneys were aseptically removed, triturated in saline and cultured for leptospira.

Six different isolates containing 10^6 organisms of *L. bataviae* were each added to duplicate tubes of liquid EMJH media containing doxycycline ranging in concentration from 0 to 8.0 mcg/ml. Decreased numbers of organisms were observed by dark field microscopic examination in tubes as concentrations of doxycycline increased. The inhibitory concentration of doxycycline was recorded when duplicate tubes had no growth at 6 weeks and no growth occurred when 0.1 ml contents from these tubes were added to fresh media not containing doxycycline and reincubated.

Three *L. bataviae* isolates were also passaged in culture to test for development of resistance to doxycycline. Using the same dilutions of doxycycline as for sensitivity testing, organisms from the tube with the highest doxycycline concentration that still permitted growth were passed to a series of new tubes containing the same increasing concentrations of doxycycline. This was repeated for a total of 5 passages. The lowest concentration of doxycycline that inhibited all growth was recorded for each passage in the series.

RESULTS

Monkeys never developed overt disease following experimental infection. However, in the first experiment 3 of 4 monkeys injected with *L. bataviae* and treated with the diluent (water), had positive blood cultures. A total

Table 1

Days of isolation of *L. bataviae* from blood, CSF and urine of infected monkeys (Experiment 1).

Treatment[a]	Blood[b]	CSF[c]	Urine[b]
Diluent			
Monkey # 18126	1, 2, 3, 4, 5, 6	7, 14	5, 6, 9, 10, 14, 21, 28
H–17	1, 2, 3, 4, 5, 6	–	5, 8, 9, 10, 21, 28, 56
G–183	1, 2, 3	–	28
G–425	–	21	–
Doxycycline			
Monkey # B–299	1, 2	–	–
H–162	1, 2	–	–
H–163	–	–	–
H–168	1	–	–

[a] Oral dose of doxycyline (4-5mg/kg) or diluent given once daily for ten days beginning the day before IP infection with 1.2×10^8 serovar *L. bataviae*.

[b] Cultured on days 0-10, 14, 21, 28, 56

[c] Cultured on days 0, 4, 7, 10, 12, 14, 21, 28, 56.

of 15 isolations from blood were made in the first 6 days post infection (Table 1). Additionally, leptospires were cultured from the CSF of 2 of these 4 monkeys after 1-3 weeks and 3 of 4 developed a leptospiruria that persisted for 4 or more weeks. The one monkey (G 425) that never developed a leptospiremia or leptospiruria had leptospires isolated from the CSF on day 21 and a titer of 1 : 512 to the *L. bataviae* serovar on day 56. In the four monkeys that were similarly infected but treated daily with doxycycline for 10 days, leptospiremia was detected only on days 1 and 2 in 3 of 4 monkeys for a total of 5 isolations. No isolates were made from the CSF or urine of the doxycycline treated monkeys.

In a second experiment 8 monkeys were treated with diluent or doxycycline 2 hours prior to infection and on day 7 (Table 2). In those receiving the diluent treatment, *L. bataviae* was isolated from the blood of each from 1 to 5 days after infection (11 positive cultures). In 2 of 4 diluent treated monkeys *L. bataviae* was cultured from the CSF and all had leptospiruria beginning 1 to 2 weeks following IP infection that persisted for 4 to 5 weeks. Two of four monkeys treated weekly with doxycycline had leptospiremia for 1 to 3 days (total of 4 positive blood cultures). No CSF isolations were obtained from doxycycline treated monkeys; one monkey in this group had a positive urine culture on day 4.

Hamsters died of leptospirosis 8-12 days following IP injection of the LC0475 isolate unless treated with doxycycline (Table 3). All hamsters treated with doxycycline even a single dose, survived. Doxycycline treatment prevented renal infection if hamsters were treated daily for 4 or more days (groups 5, 6, 7).

To support animal experiments, the sensitivity of *L. bataviae* (LC0475) to doxycycline *in vitro* was tested and compared with the sensitivity of five other isolates of that serovar.

Table 2

Days of isolation of *L. bataviae* from blood, CSF, and urine of infected monkeys (Experiment 2)

Treatment[a]		Blood[b]	CSF[c]	Urine[b]
Diluent				
Monkey #	11105	1, 2, 3	–	7, 12, 14, 18, 21, 28,35
	11093	1, 2, 3	18	7, 10, 14, 18, 21, 28, 35
	11139	1, 5	12, 14	10, 12, 14, 18, 21, 28, 35
	H-129	1, 2, 3	–	14, 18, 21, 28
Doxycyline				
	11103	–	–	–
	B-300	–	–	–
	B-432	1, 2, 3	–	4
	B-612	1	–	–

[a] Oral dose of doxycline (8-10mg/kg) or diluent given 2 hours before IP injection of 1.1×10^7 *L. bataviae* and repeated 7 days after infection.

[b] Cultured on days 0-8, 10, 12, 14, 18, 21, 28, 35, 42, 49, 56.

[c] Cultured on days 0, 4, 7, 10, 12, 14, 18, 21, 28, 35, 42, 49, 56.

Table 3

Doxycycline prophylaxis in hamsters with *L. bataviae* infection.

Group	Infection[a]	Days of treatment[b]	Number deaths/total	Positive renal culture/survivors[c]
1	LC 0475	None	5/6	1/1
2	LC 0475	−1	0/6	6/6
3	LC 0475	−1, 0	0/6	6/6
4	LC 0475	−1, 0, 1	0/6	6/6
5	LC 0475	−1, 0, 1, 2	0/4	0/4
6	LC 0475	−1, 0, 1, 2, 3, 4	0/6	0/6
7	LC 0475	−1, 0, 1, 2, 3, 4, 5, 6	0/6	0/6
8	LC 0475	−1, 6, 13	0/6	3/6
9	None	None	0/6	0/6
10	None	−1, 0, 1, 2, 3, 4, 5, 6	0/6	0/6

[a] An IP injection of 1×10^5 organisms on day 0.
[b] Oral dose of 28.8 mg/kg on day −1; 14.4 mg/kg on all other days.
[c] Cultured 6 weeks post infection.

Table 4

Growth of *Leptospira bataviae* in culture with doxycycline.

Doxycycline concentration*	Leptospira isolates					
	LC-0475**	LC-0951	LC-0691	LC-0713	p23	p13
0.0, 0.25, 0.50, 1.0	+	+	+	+	+	+
2.0	+	±	−	−	−	−
4.0, 8.0	−	−	−	−	−	−

*mcg/ml in EMJH liquid media
** + = growth in duplicate tubes; ± growth in one tube but not in duplicate; − no growth in either tube.

When doxycycline was added to media inoculated with leptospira (LC0475), no growth occurred at antibiotic concentrations of 4.0 mcg/ml (Table 4). The five other isolates of *L. bataviae* were similarly inhibited when doxycycline concentrations were 2.0 mcg/ml or greater. Repeated passage (5 times) of three isolates (LC0475, LC0118, LC0443) taken from the tube with the highest concentration of doxycycline that still permitted leptospira growth, failed to increase the concentration of doxycycline (2.0-4.0 mcg/ml) required to prevent growth in later passages.

DISCUSSION

The purpose of this study was to determine the chemoprophylactic effect of doxycycline against the *L. bataviae* serovar, a common cause of leptospirosis in Thailand. In addition to testing isolate LC0475 in two animal models, a growth inhibition test was performed

to determine the sensitivity of additional isolates *in vitro*. In non human primates, leptospirosis is generally described as an asymptomatic infection (Minette, 1966; Minette and Shaffer, 1968; Marshall *et al.*, 1980). No evidence of illness was observed in monkeys in this study although leptospira were frequently isolated from the blood, urine, and CSF of the monkeys not treated with doxycycline. *Leptospira* are also frequently present in the blood, CSF, and urine in human infection (Turner, 1967; McClain *et al.*, 1984); doxycycline prophylaxis has been shown to prevent clinical illness and leptospiruria in man (Takafugi, *et al.*, 1984; McClain, *et al.*, 1984). It is rapidly and efficiently absorbed and well distributed thoughout most tissues including kidney, brain and CSF Neu, 1978.) In monkeys doxycycline prevented leptospiruria, as well as infection of the CSF and the period of detectable leptospiremia was reduced, especially when doxycycline was given daily. The hamster model was a more critical test for the efficacy of doxycycline prophylaxis in leptospirosis. Unlike monkeys, hamsters died following *Leptospira* infection if no doxycycline treatment was given. However, protection was shown in hamsters with even a single treatment; at least 4 days of treatment was necessary to prevent chronic renal infection. It is the renal infection that is associated with kidney injury in man and animals (Sitprija *et al.*, 1980). The weekly dose schedule effective for human leptospirosis prophylaxis (Takafugi *et al.*, 1984), did not prevent chronic renal infection in all hamsters. This difference between hamsters and man may be due to the high number of organisms given to hamsters or because of different pharmacokinetics of doxycycline between species.

Doxycycline is considered bacteriostatic rather than bacteriocidal (Neu, 1978). Absence of growth on reincubation of samples from tubes where doxycycline prevented growth suggests that leptospires were no longer viable. No growth was selected as the doxycycline level for comparing sensitivity of the isolates. Although partial inhibition could be seen at lower concentrations of doxycycline, evaluation was more subjective. By *in vitro* growth inhibition testing, the sensitivity of a number of *L. bataviae* isolates not studied in animals could be compared. Other isolates were equally or more sensitive than LC0475 to doxycycline in culture. Concentrations of 2.0–4.0 mcg/ml of doxycycline in media completely inhibited growth of all isolates in culture. In man, peak blood levels exceed 2.0 mcg/ml following oral doxycycline (Leibowitz *et al.*, 1972). Serum concentrations of doxycycline attainable in man could inhibit growth of the isolates in this study.

It is not known if *Leptospira* develop antibiotic resistance. In this study no resistance developed when organisms were grown and passed in media containing concentrations of doxycycline that did not completely inhibit growth. Although there is the potential that other antibiotic resistant bacteria may emerge in the continued prophylactic use of doxycycline, the development of resistant *Leptospira* does not seem likely. We conclude that doxycycline is an effective chemoprophylactic agent against *L. bataviae* infection in animals; *in vitro* results indicate that other local isolates are similarly sensitive to doxycycline.

SUMMARY

Doxycycline was effective as a chemoprophylactic agent for experimental *Leptospira* infection in non-human primates and hamsters. Monkeys injected intraperitoneally with *Leptospira bataviae*, and receiving only diluent as treatment developed a leptospiremia during the first week and later leptospires were cultured from the cerebrospinal fluid and urine. Monkeys treated daily with oral doxycycline for 10 days beginning one day before infection had a shortened period of detectable lepto-

spiremia, and organisms were never detected in the cerebrospinal fluid or urine. Even an oral dose of doxycycline 2 hours before infection and on day 7 prevented the later infection of the cerebrospinal fluid and urine. In hamsters, doxycycline treatment prevented deaths from acute *Leptospira* infection and when hamsters were treated daily for 4 or more days, renal infection was prevented. The results of animal studies, the susceptibility of LC0475 and the five other isolates to doxycycline *in vitro*, and lack of evidence for antibiotic resistance in culture suggests this antibiotic may be useful as a prophylactic drug for high risk groups and an effective treatment for leptospirosis in Thailand.

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