Epidem. Inf. (1987), 98, 191–197
Printed in Great Britain

Experimental infection of monkeys with *Leptospira interrogans* serovar *hardjo*

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(*Accepted 3 November 1986*)

SUMMARY

Grivet monkeys experimentally infected with two different strains of *Leptospira interrogans* serovar *hardjo* showed no signs of severe clinical disease. There were no significant macroscopic lesions in any of the tissues examined, but the organisms were demonstrated in various tissues by immunofluorescent technique and were isolated from the blood and urine of two monkeys and the kidney of one. Abraded skin was shown to be a viable route of infection in non-human primates.

INTRODUCTION

Leptospirosis is a world wide zoonosis, the main reservoir of which is wild animals and in particular rodents. However, domestic animals such as dogs and farm livestock, especially cows, may also harbour the organism after becoming infected by direct contact with the leptospiral serovars specific to their host species. Thus cattle are the maintenance hosts for *Leptospira interrogans* serovar *hardjo* and the dog for *L. interrogans* serovar *canicola*. Infection may also occur by indirect contact with a serovar from another maintenance host. For example dogs may become infected with *L. interrogans* serovar *copenhageni* or *L. interrogans* serovar *icterohaemorrhagiae* from water contaminated with rat urine, or by eating infected rats. Man is infected by direct or indirect contact with the tissues, contaminated urine, blood or aborted material from a maintenance host from which the surrounding environment may become contaminated. In Great Britain the epidemiology of human leptospirosis has changed considerably over the last 20 years. In the past serovar *copenhageni* and serovar *icterohaemorrhagiae* were the prevalent strains causing infection and miners and sewer workers were the occupations at highest risk as a result of contact with rat urine (Waitkins, 1985). However, increased awareness of the problem, the use of protective clothing and effective pest control measures has resulted in a considerable decrease in the number of human cases in these groups (Anon, 1985).

Currently the most prevalent form of leptospirosis in Great Britain is that due

to serovar *hardjo* infection and dairy cattle form the major reservoir. In recent surveys 30% of all the bovine serum samples examined in the United Kingdom were positive for leptospirosis and 90% of these were attributable to serovar *hardjo* (Pritchard, 1986).

In cattle serovar *hardjo* infection has been associated with mastitis in dairy herds (Ellis *et al.* 1976*a*) and also with late pregnancy abortions (Ellis *et al.* 1976*b*). In man the disease may present most commonly as a febrile 'flu-like illness with severe headache and mental confusion or, occasionally more seriously, with meningitis with or without renal and hepatic failure. Fatalities are rare (Waitkins, 1985). Persons at risk in this country are those who are in close contact with the tissues of potentially infected animals or foetuses, e.g. farmers, vets and abattoir workers.

Recent epidemiological data collected by the Leptospira Reference Unit indicated that farm workers who suffer from the more severe meningeal form of leptospirosis have had contact with cattle herds in which abortions have occurred while the milder 'flu-like illness has been acquired from herds with leptospiral mastitis.

This study was a preliminary investigation of differences in pathogenicity of two serovar *hardjo* strains, one of which has been associated with abortion and another with milder forms of this disease. We also examined experimentally the hypothesis that contamination of abraded skin by serovar *hardjo* is a possible mode of human infection.

MATERIALS AND METHODS

Organisms

Two isolates of *L. interrogans* serovar *hardjo* were used. One strain (A), was originally described by Woods *et al.* (1983) from the kidney of an asymptomatic cow in a beef herd in Scotland. This strain is unusual in its ability to cause neurological disease in hamsters. The other strain, (B), was isolated from the aborted foetus of a Colombus monkey at Belfast Zoo by Dr W. Ellis.

Both isolates were grown in 20 ml volumes of Johnson and Harris's (Johnson & Harris, 1967) modification of Ellinghausen and McCullough's medium (EMJH) to an approximate concentration of 2×10^8 organisms/ml. The cultures were incubated at 30 °C for 5-7 days prior to inoculation. The numbers of organisms/ml were determined using a Thoma counting chamber.

Animals

A total of eight adult grivet monkeys (*Cercopithecus aethiops*) of either sex and weighing 4·5–6·2 kg were used. They were caged singly and fed on pelleted diet ('Mazuri' Special Diet Services) with additional fresh fruit daily and water *ad libitum*.

Inoculation and sampling procedures

For all procedures, including the taking of rectal temperatures, the monkeys were anaesthetized by the intramuscular injection of ketamine hydrochloride ('Vetlar', Parke-Davis). Blood samples were taken from the femoral vein. In the initial study monkey no. 1 was inoculated subcutaneously and intraperitoneally

with strain A and monkey no. 2 with strain B (1 ml into each site). The remaining animals, numbered 3 to 8 were infected by skin scarification. Monkey 3 was inoculated with strain A and monkeys 4–8 were inoculated with strain B. A site approximately 3 × 3 cm in the middle of the back was clipped, shaved, swabbed with surgical spirit and scarified with the tip of a sterile needle. One ml of the organism was then poured onto the abraded skin and left to dry. The infecting dose of leptospires/ml in EMJH was 2×10^8 organisms for each inoculation (except monkey 8, which was inoculated with homogenized kidney from monkey 7, the inoculating dose in this case being unknown).

Serological and cultural techniques

Although 8 monkeys were used for histopathology, only 6, numbers 3 to 8, were examined by bacteriological and serological methods.

Culture of tissue and body fluids. The technique used was previously described by Ellis, O'Brien & Cassells (1981).

Blood. Samples of blood obtained at intervals throughout the experiments were serially diluted in 1 % bovine serum albumin diluent (BSAD) (Ellinghausen, 1973). One ml volumes of blood were added to 9 ml volumes of BSAD and 10-fold dilutions were made (10^{-1}–10^{-5}). Volumes of 50 to 100 μl of these dilutions were then inoculated into two different media. One medium consisted of 5 ml volumes of EMJH containing 0·1 % agar and 1 % rabbit serum, the other was additionally supplemented with 5-fluorouracil (100 μg/ml). One ml volumes of blood from monkeys 5 and 6 were also inoculated into 5 ml volumes of 1 % liquoid on days 1, 2, 3, 6, 8 and 14 after infection. These were further subcultured into culture medium as described.

Urine. Samples were taken at intervals from 2 days to 12 weeks after infection and subcultured as described for blood. Urine samples were obtained by catheterization of the bladder under general anaesthesia using a size 3FG Cat Catheter (Arnolds Veterinary Products).

Tissue. At *post mortem*, organs and biological fluids were aseptically removed, 1 g portions of tissue were mechanically homogenized in 9 ml volumes of BSAD and cultured as before. Cerebrospinal fluids and aqueous humour samples were diluted in 10-fold steps from neat to 10^{-4} prior to culture. All inoculated media was incubated at 30 °C for 16 weeks and examined at 2-week intervals by dark ground illumination for the presence of leptospires. All fluids and tissues removed throughout the experiments and at necropsy were cultured within 1 h of removal.

Necropsy procedures. Animals were killed at 11 and 17 days, and 4, 5, 6, 8 and 12 weeks after infection by intravenous injection of pentobarbitone sodium. Portions of the following organs were taken and fixed in 10 % buffered neutral formalin for histopathological examination; brain, spinal cord, lung, myocardium, eye, liver, kidney, spleen, gastro-intestinal tract, bladder, thigh muscle, axillary and mesenteric lymph nodes. After processing by standard methods the tissues were embedded in paraffin wax and sections cut at 5 μm. Sections were stained with haemotoxylin and eosin and selected sections were also stained by Warthin–Starry method and by the periodic acid–Schiff technique.

Serology. Leptospiral antibodies were measured using the microscopic agglutination test (MAT) as described by Turner (1968).

Table 1. *Antibody titres to serovar* hardjo-*infected monkeys (Microscopic Agglutination Test)*

		Monkey no.					
		3	4	5	6	7	8
Day no.	0	0	0	0	0	0	0
	8	0	0	NT	NT	NT	NT
	11	160	40	NT	NT	NT	NT
	14	160	640	640	640	NT	NT
	17	160	640	NT	NT	NT	NT
	21	320	1280	NT	NT	NT	NT
Week no.	4	NT	NT	NT	NT	NT	0*
	5	NT	NT	40*	320	NT	—
	6	NT	2560*	—	NT	NT	—
	8	NT	—	—	NT	160*	—
	12	320*	—	—	0*	—	—

Monkey no. 3: Infected with Woods strain of *hardjo* (Strain A).
Monkey no. 4: Infected with aborted foetus isolate of *hardjo* (Strain B).
Monkeys nos. 5–7: Infected with aborted foetus isolate of *hardjo* from kidney of Monkey 4.
Monkey no. 8: Infected directly with kidney homogenate from Monkey 7.

* Day killed. NT, not tested

Immunofluorescence. Tissue homogenates and body fluids were examined for the presence of leptospires using the technique of Ellis *et al.* (1982) without counterstaining.

RESULTS

Clinical and necropsy findings

All infected monkeys were dull and anorexic up to 4 days after infection. Slight elevations of temperature occurred intermittently for up to 10 days in the six given the abortion strain (0·6–2 °C rise). Thereafter all animals remained normal.

Serology

The results are presented in Table 1. Monkey 3 inoculated with strain A had a titre to *hardjo* of 320 when killed at 12 weeks (Table 1). Monkeys 4, 5, 6 and 7 all produced varying antibody responses ranging from 2560 in the case of Monkey 4 to 160 in Monkey 7 (Table 1). Monkey 8 had no antibody when killed at 4 weeks.

Bacteriological findings

Table 2 shows the culture results from tissues and body fluids. Monkey 3, inoculated with strain A, was negative by culture for all specimens. However three of the monkeys inoculated with strain B (nos. 4, 5, 6, 7 and 8) were culture positive. Serovar *hardjo* was isolated from the urine of monkey 4 on day 8 and from its kidneys after it was killed at 6 weeks post inoculation. No leptospires were grown from the tissues of monkeys 5 and 6 at necropsy though blood cultures from these animals had been positive for leptospires on days 1 and 2 after infection. The duplicate blood samples incubated at 30 °C in 1% liquoid for approximately 2

Table 2. *Isolation of serovar* hardjo *from monkey tissues and body fluids*

	Monkey no.					
	3	4	5	6	7	8
Kidney	—	+	—	—	—	—
Spleen	—	—	—	—	—	—
Brain	—	—	—	—	—	NT
Bladder	—	NT	—	—	—	NT
Liver	—	—	—	—	—	—
Lung	—	—	—	—	—	—
Aqueous humour	—	—	—	—	—	—
Bile	—	—	—	—	—	—
CSF	—	NT	—	NT	—	NT
Urine	—	(day^{+}8)	—	NT	—	NT
Blood	—	—	(days^{+} 1, 2)	(days^{+} 1,2)	—	—

—, Negative culture. +, Positive culture. Day, day sample taken. NT, Not tested. CSF, Cerebropinal fluid.

Table 3. *Detection of serovar* hardjo *by direct IF in monkey tissue homogenates and body fluids*

	Monkey no.					
	3 (12 wk)	4 (6 wk)	5 (5 wk)	6 (12 wk)	7 (8 wk)	8 (4 wk)
Kidney	—	+	+	—	+	+
Spleen	—	+	+	—	—	+
Brain	—	+	+	+	—	NT
Bladder	—	NT	—	—	—	NT
Liver	—	+	—	+	—	+
Lung	—	+	—	—	+	+
Aqueous humour	—	—	—	—	—	—
Bile	—	—	—	—	—	—
CSF	—	NT	—	NT	—	NT
Urine	—	—	—	NT	—	NT
Blood	NT	NT	NT	NT	NT	NT

—, Negative. +, Positive. NT, Not tested. CSF, Cerebrospinal fluid.

weeks prior to culture were also positive. All tissues and body fluids of monkeys 7 and 8 were negative by culture for serovar *hardjo*.

Immunofluorescence microscopy

All tissues and fluids from Monkey 3 were negative by fluorescence (IF). Leptospires were detected however, by IF in various tissues from all the monkeys inoculated with the abortion-associated strain (Table 3).

Histopathology

There were no lesions in organs other than the kidney in any of the animals. Mild changes were present in six of the monkeys from 11 days to 12 weeks after infection (monkeys 2, 4, 5, 6, 7 and 8). Foci of lymphoid cell infiltration were

scattered throughout the renal cortex, at the cortico-medullary junction and in the medulla, and were most conspicuous at 5–6 weeks. The lymphocytes infiltrated between tubules, some of which contained PAS-positive amorphous material. Leptospires could not be demonstrated in sections by the Warthin–Starry technique.

DISCUSSION

This study has established that serovar *hardjo* can cause systemic infection in a non-human primate following contamination of abraded skin and supports the impression gained from epidemiological investigations that human leptospirosis may possibly be contracted from cattle by this route.

The disease induced in monkeys using either strain was clinically mild, fever being only moderate and of short duration, even though bacteraemia developed and there were focal lesions in the kidneys in some of the monkeys inoculated with strain B. The disease mimicked the mild but not the severe form of human *hardjo* infection despite the use in some experiments of a strain inducing abortion in animals. This may have been due to an inherently greater resistance of these monkeys or because the organisms had become attenuated during *in vitro* cultivation.

Bacteraemia was proven by culture in two monkeys on days 1 and 2, and further dissemination of the bacteria to major organs, including the brain, was demonstrated by immunofluorescence in several monkeys at different stages of infection. The inability to culture serovar *hardjo* from tissues, although organisms were demonstrable in the same organs by immunofluorescence, has been reported by others before (Ellis, 1980; Smith, Reynolds & Clark, 1967). It is suggested that this may be due in part to the fastidious nature of the growth requirements (Ellis & Michna, 1976) and in part to the inhibition of leptospiral growth in the presence of tissue autolysis (Smith, Reynolds & Clark, 1967). The cultural methods employed may not have been sufficiently sensitive to detect very small numbers of organisms or perhaps the organisms demonstrated by immunofluorescence were non-viable. No cellular inflammatory reaction was found in brain or liver sections taken from areas in which organisms were demonstrated by immunofluorescence in smears. Though the leptospires had clearly persisted for up to 12 weeks multiplication may have been inadequate to provoke a response. There was no histological evidence of meningitis in any of the animals, in contrast to the findings in grivet monkeys experimentally infected with *L. interrogans* serovars *balcanica* and *tarassovi* (Marshall *et al.* 1980). The uncertainties about the virulence of our strains make comparisons difficult to interpret and further work using freshly isolated bovine strains of *L. hardjo* from cases of mastitis and abortion would produce a more realistic model for interpretation of human infections.

In this study we have demonstrated that the grivet monkey (*Cercopithecus aethiops*) may be a suitable animal model for the study of serovar *hardjo* infections in man and produces a similar clinical response as found in milder human infections (Waitkins, 1985).

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