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BENIGN LEPTOSPIROSIS: THE PATHOLOGY OF EXPERIMENTAL INFECTION OF MONKEYS WITH *LEPTOSPIRA INTERROGANS* SEROVARS *BALCANICA* AND *TARASSOVI*

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Summary.—Grivet monkeys experimentally infected with *Leptospira interrogans* serovars *balcanica* and *tarassovi* showed no clinical disease, but severe meningoencephalitis was demonstrated histologically in animals killed 26 and 33 days after infection. The meningeal and perivascular reactions were exclusively lymphocytic. Mild focal lesions of degeneration and cellular infiltration were also present in the kidneys and femoral musculature.

LEPTOSPIROSIS is a widely prevalent zoonosis of increasing importance throughout the world (WHO, 1972) and outbreaks of human disease are not infrequently reported from Britain (Seiler, Norval and Coghlan, 1956; Alston and Broom, 1958), Europe (Levrat *et al.*, 1950; Austoni and Cora, 1960; Fenske, Fenske and Oberdoerster, 1969), the United States (Ward *et al.*, 1956; Heath, Alexander and Galton, 1965; Fraser *et al.*, 1973), New Zealand (Christmas *et al.*, 1974*a*,*b*; New Zealand Department of Health, 1978), and the Far East (Berman *et al.*, 1973).

Because benign human leptospirosis, *i.e.* those infections due to serovars other than *canicola* and *icterohaemorrhagiae*, is almost never fatal there has been little opportunity to study the pathology of these infections. Clinical signs and symptoms suggest that involvement of the central nervous system often occurs during the acute phase (Gsell, 1978). The evidence includes the frequent occurrence of severe headaches and the occasional reporting of schizophreniform psychoses (Avery, 1976; Marshall and Scrimgeour, 1978) and confused states. Unpublished observations also indicate a period of convalescence for benign leptospirosis which extends well beyond that which might be anticipated from the relatively mild clinical features of the acute phase.

This study therefore set out to define the nature and extent of pathological changes associated with benign leptospirosis as exemplified by serovar *balcanica* and *tarassovi* infections in a non-human primate. Experimental leptospirosis has been studied using other serovars (Minette and Schaffer, 1968) but their observations were confined to clinicopathological and serological changes.

MATERIALS AND METHODS

Animals.—Thirteen adult Grivet monkeys (*Cercopithecus aethiops*) of either sex were used. One animal served as an uninfected control and received by i.m. injection 1 ml of culture medium only. Six monkeys were inoculated i.m. in the lateral aspect of the upper left arm with 1 ml of a 5–7-day culture containing $5{\cdot}1 \times 10^8$ *Leptospira interrogans* serovar *balcanica* organisms, and a further 6 animals were given 1×10^8 *L. interrogans* serovar *tarassovi* bacteria in a similar manner. For inoculation, nasal swabbing, temperature recording and blood sampling the monkeys were anaesthetized by i.m. injection of ketamine hydrochloride (Vetalar—Parke, Davis).

Growth of leptospirae.—For growth of both serovars 0·3 ml of a 5–7-day culture (containing

approximately 1×10^8 organisms per ml) was inoculated into 5 ml of Johnson–Harris Tween 80 medium supplemented with bovine albumin Fraction V (Miles Laboratories, U.S.A.). The culture was incubated at 30° for 5–7 days before infection of the monkeys. Leptospiral counts were assessed using a Hawksley bacterial counting chamber. Preliminary experiments showed that i.p. inoculation of *balcanica* killed 6/6 weanling hamsters and 3/5 died after similar infection with *tarassovi*.

The isolates used, *balcanica* and *tarassovi*, were originally recovered respectively from a possum (*Trichosurus vulpecula*) and a pig in New Zealand, and identified by cross-agglutination absorption at the WHO Reference Laboratory Centre for Disease Control, Atlanta, U.S.A.

Procedure for isolation of leptospirae from samples

Blood.—0·04 ml of heparinized blood, obtained by femoral venepuncture, was added to each of two separate culture bottles containing 5 ml of Johnson–Harris Tween 80 medium supplemented with 1% bovine albumin Fraction V and 0·2% Oxoid No. 1 agar. One of each culture bottles was additionally supplemented with 200 μg/ml 5-fluorouracil (5 fu) (Sigma Chemical Corp., U.S.A.).

Tissues.—At necropsy organs and fluid samples were aseptically removed, washed with industrial spirit and flamed to reduce surface contamination. Portions of each organ (brain, kidney, liver, left axillary lymph node, and thigh muscle) were mechanically homogenized in 10ml aliquots of Stuart's medium and these specimens and aqueous humour, urine and cerebrospinal fluid were then further diluted 1:10 with Stuart's medium. Aliquots of 0.12 ml of both homogenized and 1 in 10 homogenized tissue suspended in Stuart's medium were then added to each of 3 5ml aliquots of Johnson–Harris Tween 80 medium supplemented with bovine albumin Fraction V and agar. Each of the culture media contained the following amounts of 5 fu:

1. Johnson's medium + 0 μg/ml 5 fu.
2. Johnson's medium + 200 μg/ml 5 fu.
3. Johnson's medium + 400 μg/ml 5 fu.

All culture bottles were incubated at 30° and examined at 1, 3, 6 and 12 weeks later.

Necropsy procedure

One monkey from each infection group was killed by i.v. injection of pentobarbitone sodium at each of the following stages after infection: 7, 18, 26 and 33 days; and 2 animals from each group were killed on the 10th day. The control monkey was killed at 33 days. At necropsy portions of the following organs were removed from each monkey for histopathology: brain, cervical spinal cord, pharynx, palatine tonsil, trachea, lungs, myocardium, mediastinal and mesenteric lymph nodes, spleen, liver, stomach, ileum, caecum, colon, kidney, bladder, and anterior, medial and posterior muscles of the thigh. The tissues were immediately fixed in 10% buffered neutral formalin, processed and embedded in paraffin wax by standard methods. Sections of all tissues were cut at 5 μm and stained with haematoxylin and eosin. Selected sections were stained by the periodic-acid–Schiff technique, by Verhoeff–van-Gieson, Mallory's phosphotungstic-acid–haematoxylin, by Gordon and Sweet's method, and by Young's silver impregnation technique for leptospirae (Young, 1969).

Kidney samples only were processed for electron microscopy. Small portions of renal cortex tissue were excised from the 33-day animals and fixed for 2 h in cold 4% cacodylate-buffered glutaraldehyde, stained with 1% buffered osmium tetroxide, processed by standard methods and embedded in Araldite. For identification of suitable tissue areas 1μm-thick sections were cut and stained with Toluidine Blue. Ultra-thin sections were prepared using a Reichert Ultramicrotome.

Serology

The monkeys were bled just before inoculation and on Days 3, 4, 5, 6, 7, 10, 12, 14, 17, 21, 26 and 33 after inoculation with leptospiral culture or until the day of killing. The microscopic agglutination test (MAT) was performed using a modification of the microtitre plate technique previously described by Cole, Sulzer and Pursell (1973). The lowest dilution used was 1:24 and the titre is expressed as the highest dilution showing at least 50% agglutination.

RESULTS

Gross necropsy findings

Lesions were detected in only the brains of monkeys infected with *balcanica* and *tarassovi* at 26 and 33 days after infection. The changes were seen as white opaque areas of the meninges covering the gyri and in the sulci and fissures of the cerebral hemispheres. There were no other gross lesions.

Histopathology

Lesions were confined to the kidneys of those monkeys infected with *balcanica* and *tarassovi* and killed at 7, 10, 18, 26 and 33 days, to the brains of the animals infected

with both serovars for 26 and 33 days, and to the quadriceps femoris group of muscles of some of the *tarassovi*-infected animals.

Kidneys.—The renal changes consisted of small randomly scattered foci of lymphoid infiltration (Fig. 1). These were situated predominantly in the outer cortex but some were also present at the cortico–medullary junction. Neighbouring tubules showed vacuolation, degeneration and necrosis of epithelial cells but the amount of debris in tubular lumina was generally

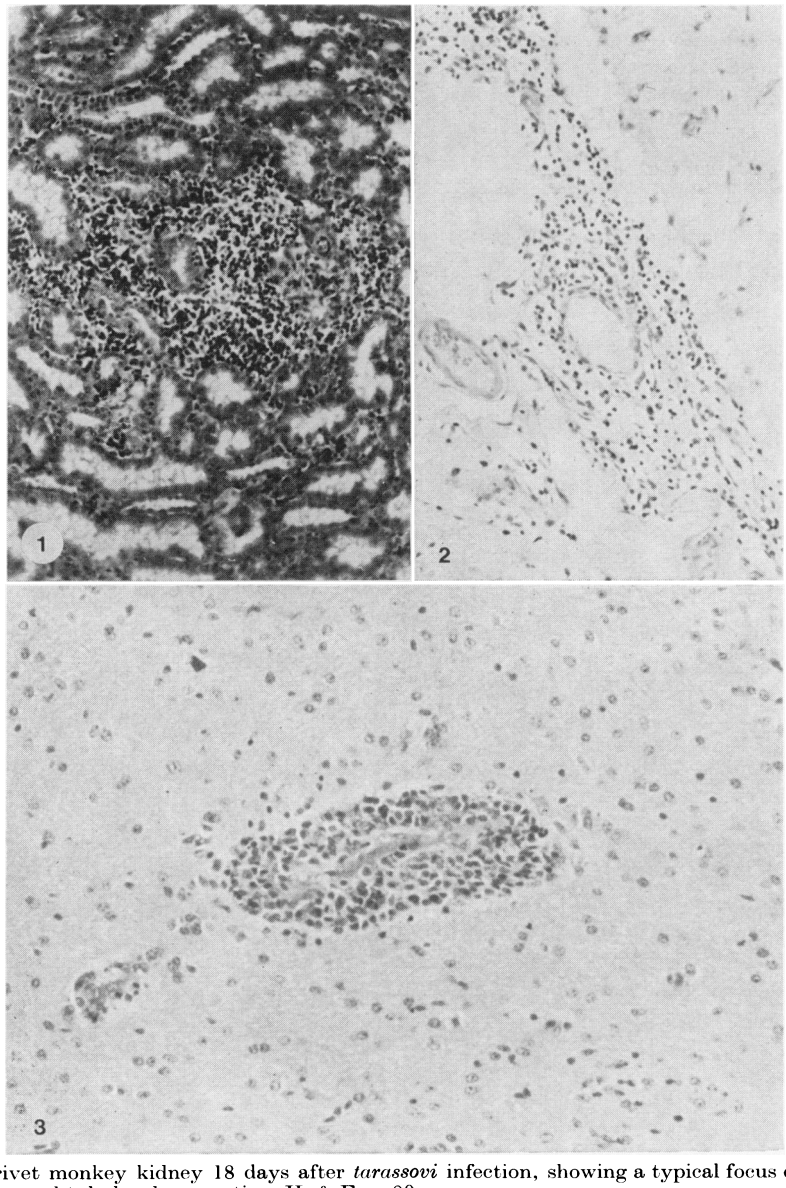

FIG. 1.—Grivet monkey kidney 18 days after *tarassovi* infection, showing a typical focus of lymphoid infiltration and tubular degeneration. H. & E. × 80.

FIG. 2.—Brain of *tarassovi*-infected monkey at 26 days. There is heavy infiltration of the cerebral cortical meninges by lymphocytes. H. & E. × 70.

FIG. 3.—Perivascular cuff of lymphocytes in cerebral cortex of *tarassovi*-infected monkey, 26 days. H. & E. × 125.

slight. Small numbers of glomeruli had adhesions between the capillary loops and the parietal layer of Bowman's capsule. Bowman's space also contained variable quantities of proteinaceous material. The lymphoreticular foci were larger in the monkeys killed at 26 and 33 days than at the earlier stages and contained more plasma cells and minimal fibrosis. No difference in the nature of the lesions was apparent between those caused by *L. balcanica* and *L. tarassovi*.

Examination of kidney sections stained by Young's silver impregnation method demonstrated small clumps of leptospirae in the lumina of proximal convoluted tubules and in tubular epithelium in 5 of 12 infected monkeys (7-, 10- and 18-day *L. tarassovi* infection, 10- and 33-day *L. balcanica* infection).

Brain.—Lesions were present only in monkeys killed at 26 days or later. The changes were those of a non-suppurative meningo-encephalitis. They were most severe and extensive in the *tarassovi*-infected monkey killed 26 days after infection, but similar changes were also present in the *balcanica*-infected monkey killed at the same stage. Lesions consisted of diffuse and focally heavy infiltration of the meninges by large and small lymphocytes and occasional plasma cells (Fig. 2). Polymorphonuclear leucocytes were absent from the inflammatory population. The cellular infiltrates involved most cerebral gyri and extended deeply into the cerebral fissures and sulci. The cerebellar meninges were only mildly involved. In the cerebral cortex and mid-brain some microglial activation was taking place in the grey matter immediately below the meningeal infiltrations.

Perivascular cuffs of lymphocytes were a prominent feature in the brains of the two 26-day monkeys and were present throughout the grey and white matter of the cerebral cortex, caudate nucleus, putamen, hippocampus and mid-brain. The perivascular cuffs varied in thickness from 1 or 2 cells to as much as 8 cell layers deep (Fig. 3). Affected blood vessels frequently exhibited endothelial swelling and damage, and were partially occluded by cells and debris. A few regions of the cerebral cortex and mid-brain contained areas of diffuse and focal microgliosis but necrosis of neurones was rare. Diffuse and focal infiltration of the choroid plexuses by lymphocytes was present in many areas and the plexus of the IVth ventricle was particularly affected. Lesions in the substance of the cerebellum were not found.

Of the 2 monkeys infected with *balcanica* and *tarassovi* and killed at 33 days, both had residual meningeal cellular infiltrations and small perivascular cuffs in the brain substance. The lesions had resolved considerably and were much smaller than those present in the 26-day animals. However, lymphocytic aggregations in the choroid plexus of the IVth ventricle were still prominent in the *tarassovi*-infected monkey.

It was not possible to identify leptospirae in any of a large number of brain sections stained by Young's silver impregnation method. Location of the organisms was made extremely difficult by the similar staining properties and size of brain fibres.

Cervical spinal cord.—Lesions were not present in the cervical spinal cord.

Femoral musculature.—In the muscles of the antero-medial aspect of the thigh in *tarassovi*-infected monkeys at all stages from 10 days there were small foci of lympho-histiocytic infiltration, mild sarcolemmal proliferation and hyaline degeneration and loss of striation of occasional fibres.

Other organs.—Significant lesions were not present in other tissues nor in those of the uninfected control animal.

Electron microscopy

Although loss of the brush border and even some necrosis of proximal and distal tubular epithelium was present, leptospirae were not found in the small number of kidney blocks taken from *balcanica* and *tarassovi*-infected monkeys at 33 days.

TABLE I.—*Antibody titres in* tarassovi-*infected monkeys*

Monkey No.:	14		3		4		24		11		5	
Day	t	b	t	b	t	b	t	b	t	b	t	b
0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
4	0	24	0	0	0	0	0	24	0	0	0	0
5	0	0	48	48	48	24	192	96	48	0	48	24
6	48	96	24	48	96	48	192	48	192	0	192	0
7	96	48	48	24	192	24	192	24	96	0	96	24
10			96	48	384	192	192	48	192	192	384	48
12							768	96	192	96	192	24
14							768	384	192	192	96	24
17							768	384	192	96	48	24
21									96	96	192	0
26									48	96	48	0
33											48	0

t = *tarassovi* antigen.
b = *balcanica* antigen.

TABLE II.—*Antibody titres in* balcanica-*infected monkeys*

Monkey No.:	75		B16		16		18		A9		B20	
Day	t	b	t	b	t	b	t	b	t	b	t	b
0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	24	0	0	ND	ND	0	24	0	24
5	0	96	0	96	0	96	0	48	0	48	0	96
6	0	96	0	192	0	192	0	96	0	192	0	96
7	0	48	0	192	ND	ND	0	192	0	192	0	192
10			0	384	0	192	48	192	0	192	96	384
12					0	384	0	0	0	192	ND	ND
14					0	192	ND	ND	0	192	0	96
17					0	192	48	768	0	96	0	96
21									0	96	0	96
26									0	192	0	96
33											0	48

ND = not done (serum not collected).
t = *tarassovi* antigen.
b = *balcanica* antigen.

Bacteriological findings

Leptospirae were isolated from blood samples taken from Monkey B20 (*balcanica*) and Monkey 18 (*balcanica*) 3 days after infection. *Tarassovi* was isolated from the heart blood of Monkey 24 killed 18 days after infection. *Balcanica* was cultured at necropsy on Day 33 from the urine and liver of Monkey B20. Other body fluids and organs of all other animals were negative for leptospirae, despite culture for 12 weeks.

Serological responses

The serological responses to both *tarassovi* and *balcanica* antigens employed in the MAT are shown in Tables 1 and 2.

DISCUSSION

The disease produced in Grivet monkeys by infection with *balcanica* and *tarassovi* was subclinical, though significant changes did occur in a wide range of serum biochemical parameters (Hambleton *et al.*, 1980).

It is of particular interest that the severe and widespread meningo-encephalitis demonstrated histopathologically in the monkeys in the later stages of the disease did not present clinically apparent manifestations. Headache is a consistent feature of human infection with most serovarieties of leptospirae, such as *copenhageni*, *canicola*, *pomona* and others (Beeson and Hankey, 1952; Arean, 1962;

Berman *et al.*, 1973; Fraser *et al.*, 1973). However, the detection of such a subjective sign as headache in experimental animals is not possible unless it is accompanied by dullness, inappetence or severe systemic malaise. Meningitis is common in human leptospiral infection and in many cases may be responsible for the headache experienced in the absence of fever. A number of clinical surveys have found meningitis to be present in 40% of cases in Great Britain (Alston and Broom, 1958) and in up to 68% of those in the United States (Heath *et al.*, 1965). It is surprising that meningo-encephalitis was not seen in animals killed earlier than 26 days after infection, though this is in accord with the assumed incubation period in human leptospiral disease of 10–21 days (Berman *et al.*, 1973) and the observations of Gsell (1949) that meningeal symptoms tend to develop in the later stages of the disease.

The meningeal cellular infiltration and perivascular cuffing reaction in the brain substance were exclusively lymphocytic, and the polymorphonuclear leucocytes normally present in bacterial infections of the central nervous system were not a feature. This finding correlates well with the predominance of lymphocytes in the cerebro-spinal fluid in non-fatal human leptospiral meningitis (Berman *et al.*, 1973). Arean (1962), in a survey of fatal human cases of Weil's Disease, found lymphocytic perivascular infiltrations commonly present throughout the brain, but in contrast to our findings there was no meningitis. The brains of monkeys killed 33 days after infection showed considerable resolution of the lesions as compared with those at 26 days. This, together with the absence of neuronal destruction, indicates that the brain damage was not permanent and explains well the clinical recovery of human patients from those leptospiral infections in which hepatic and renal failure is not a major feature.

In the anterior and medial thigh muscles of the monkeys there were mild lesions of focal hyaline degeneration of fibres, loss of cross striations, localized lymphocytic infiltration and sarcolemmal nuclear proliferation. Elevated levels of serum creatine phosphokinase were found in the animals (Hambleton *et al.*, 1980) and are probably the result of these changes. Myalgia, particularly of the legs, is a common clinical sign of human infection with virtually all serotypes of leptospirae (Berman *et al.*, 1973; Fraser *et al.*, 1973), even during afebrile phases. Our results suggest that actual lesions in the muscles may be responsible for the myalgia.

Macroscopic lesions were absent from the kidneys of the monkeys in both the *balcanica*- and *tarassovi*-infected groups. However, microscopic foci of lymphoreticular infiltration and tubular degeneration in the cortex were present, though the degree of renal damage was slight and there was no biochemical evidence of renal dysfunction (Hambleton *et al.*, 1980). The occasional, mild nature of the kidney lesions and clinical disease and the frequent absence of macroscopic changes in the kidneys have also been noted in pigs infected with *canicola* and *copenhageni* (Michna and Campbell, 1969) and in dogs infected with *canicola* (McIntyre and Montgomery, 1952; Taylor, Hanson and Simon, 1970). Renal lesions are of varying severity in human infection with *copenhageni* and *canicola* and other leptospirae (Arean, 1962; Brito *et al.*, 1965) and in man, cattle and dogs the degree of renal impairment is frequently the main factor governing recovery or death (Sheehan, 1946; Wylie, 1946; Ristic *et al.*, 1957; Brito, 1968; Keenan, Alexander and Montgomery, 1978).

Although isolation of *balcanica* was made from the liver of a single monkey killed at 33 days, hepatic lesions were not found histologically in this or other animals. The liver would thus appear not to be a target organ for these serovars in primates, as it is in infection with *copenhageni* and *canicola*.

In general our isolation success from tissue was poor. Leptospirae were not isolated from the brain, cerebro-spinal fluid or aqueous humour at any stage of

the infection, a result which has also been recorded by other workers using the dog as the experimental animal (Keenan *et al.*, 1978). The organisms may be present in the central nervous system only transiently and may have been missed in our experiment because of the 7–8-day killing intervals. Leptospiraemia was present in 2 of the *balcanica*-infected monkeys 3 days after infection and *tarassovi* was isolated from the heart blood of a monkey killed 18 days after infection. Leptospiraemia in human infection can be intermittent, but usually occurs for only a few days (Berman *et al.*, 1973).

A feature of the serological results is the fact that all the *tarassovi*-infected monkeys had demonstrable titres to *balcanica* at some stage during the study. These heterologous reactions were either equal to, or within one dilution of, the homologous titres on nearly 50% of occasions. This is in contrast to the *balcanica*-infected monkeys, where only 2 (18 and B20) had *tarassovi* titres and these were on isolated occasions. A possible explanation for such a reaction is inadvertent contamination with *balcanica* of the *tarassovi* culture used to inoculate the monkeys in this experiment. Cultures used were, as far as could be determined, pure, although the possibility that a very small number of *balcanica* organisms were present cannot be ruled out entirely. Every care was taken to prevent such an occurrence and the identity of the culture was confirmed serologically. Other workers have commented on the difficulties of proving dual leptospiral infections which may occur naturally. An alternative explanation of these results is that *tarassovi* infection in the Grivet monkey induces a heterologous response, suggesting that *tarassovi* and *balcanica* possess a common antigen. The titres which developed in the monkeys were not particularly high, the highest titre being 768 in two monkeys, and the range of titres was the same for both serovars. The earliest stage at which any serological response was noted was on Day 4.

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