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Experimental infection of Rhesus monkeys with a human strain of *Campylobacter jejuni*

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SUMMARY

Young Rhesus monkeys (*Macaca mulatta*) were infected orally with a human strain of *Campylobacter jejuni*. The disease induced was mild, with inappetence and diarrhoea of short duration, but prolonged intermittent excretion of the bacteria in the faeces occurred. Bacteraemia was generally present for 2–3 days and later the organisms localized in the liver and gall bladder. Recovered animals, when challenged with the same strain, showed no clinical symptoms, no bacteraemia, and excreted the organisms in the faeces for only 3 days.

INTRODUCTION

In recent years there has been increasing recognition of the role of *Campylobacter jejuni* (Veron & Chatelain, 1973) as a cause of enteritis in man (King, 1957; Wheeler & Borchers, 1961; Dekeyser *et al.* 1972; Cadranel *et al.* 1973; Skirrow, 1977; Butzler, 1979; Itoh *et al.* 1980). Although the epidemiology of the infection is poorly understood, many appear to result from ingestion of contaminated poultry or milk (Grant, Richardson & Bokkenheuser, 1980; Blaser *et al.* 1979; Robinson *et al.* 1979). An understanding of the pathogenesis of *C. jejuni* infection and its natural history is extremely important for the control of this disease. To date there have been relatively few attempts to infect laboratory animals with strains of *C. jejuni* known to be pathogenic for man. This report describes the experimental infection of Rhesus monkeys with a strain of *C. jejuni* originally isolated from an outbreak of diarrhoea and vomiting in children and thought to have been transmitted by infected cow's milk (Dr A. T. Willis, personal communication). The aims were to determine whether infection could be established, the persistence and excretion of bacteria, the clinical effects, the sites at which the organisms localized, and the lesions produced.

MATERIALS AND METHODS

Campylobacter culture

A strain of *C. jejuni* designated V212X isolated from the milk of a cow which had been experimentally infected with a human isolate (Lander & Gill, 1980) was grown under microaerobic conditions (see Media Section) in nutrient broth containing 7% sterile horse blood at 42 °C for 18 h. Standardization of inoculum was obtained by a total count of formalinized organisms (red cells removed by differential centrifugation) using a Helber slide under dark-ground illumination. Colony counts on the inoculum were used to confirm numbers of viable organisms administered. Total and viable counts were found to be approximately equal.

Experimental animals and inoculation

Eight laboratory bred Rhesus monkeys (*Mucaca mulatta*) (5 male, 3 female) between one and two years old and weighing 2–2·5 kg were used. For infection and blood sampling the animals were anaesthetized by intramuscular injection of ketamine hydrochloride ('Vetalar', Parke, Davis & Co.). Six monkeys were infected by the oral instillation of 8 ml broth containing 1×10^9 *C. jejuni* from a two day culture of the organism, and two received 1×10^{10} organisms. These last two animals were challenged orally 15 weeks later with 1×10^9 organisms. Blood samples were taken from the femoral vein for bacteriological culture daily from days 0–4 and 7–9 and at intervals up to 46 days after infection. Rectal temperatures were recorded for 10 days after infection.

Before infection, the faeces of all eight monkeys were screened for the presence of *C. jejuni* and other campylobacter for three weeks and were found to be negative. One monkey was killed at each of the following stages after infection; 3, 7, 14, 21, 30 and 46 days. The remaining two monkeys were retained for challenge and long-term studies. Before necropsy, each animal was deeply anaesthetized for a short period by the intravenous (i.v.) injection of pentobarbitone sodium and portions of the ileum were ligated and fixed for subsequent processing for histology by the injection into the lumen of 10% buffered neutral formalin. The monkey was then killed by i.v. injection of an overdose of barbiturate. At necropsy, immediately after death, the following tissues were taken aseptically for bacteriological culture and histopathological examination: palatine tonsil, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, gall bladder, liver, spleen, kidney, urinary bladder, urine, mesenteric lymph nodes. The brain was removed for histology. All tissues for histology were fixed in 10% buffered neutral formalin, processed by standard methods and embedded in paraffin wax. Sections cut at 5 μm were stained with haematoxylin and eosin, and selected sections were stained by Gram's method and by the Warthin–Starry silver impregnation technique.

Culture Media

The solid medium (Campylobacter agar) used was a modification of the selective medium of Skirrow (1977); it comprised 7% lysed horse blood agar containing vancomycin (10 μg/ml), trimethoprim (5 μg/ml), polymyxin B (2·5 i.u./ml) and actidione (100 μg/ml).

Liquid media included nutrient broth (Oxoid Ltd.) and a selective medium comprising Veal Infusion Broth (Difco Laboratories) plus 7% lysed horse blood, 1% Bacterial Charcoal (Oxoid Ltd), Vancomycin (40 μg/ml), trimethoprim (20 μg/ml), polymyxin B (10 i.u./ml), actidione (100 μg/ml) and 5-fluorouracil (500 μg/ml) (K. P. Lander, to be published). The liquid selective medium was dispensed in volumes of 5 ml in screw-topped bijou bottles.

Campylobacter Isolation

Tissues were placed in sterile polythene bags together with 5 ml of saline and macerated for 1 min in a Colworth Stomacher 400 (Model BA 6021). Maceration of tough tissues was completed by grinding in a mortar and pestle with sterile washed sand. After maceration, 0·5 ml portions of each sample were placed in the selective medium, anaerobically incubated at 42 °C for 18–24 h, and then subcultured on to Campylobacter agar. Plates were incubated in a microaerobic atmosphere produced by using a Gaspak Anaerobic system (BBL, Division of Becton, Dickinson & Company) without a catalyst, at 42 °C for 18 h.

Blood specimens were collected into Heparin (Boots Company Ltd) to a final concentration of 5 units/ml and added to nutrient broth to a concentration of 5% v/v incubated microaerobically at 42 °C for 18 h. The cultures were then plated on to Campylobacter agar.

Urine samples were plated directly on to Campylobacter agar. Faeces samples (0·06 g) were placed directly into selective enrichment medium and treated as for macerated tissue.

C. jejuni was recognized by its characteristic spiral or s-shaped morphology, Gram-negative staining reaction, and the appearance of colonies which were low convex or flat, effuse, greyish in colour in incident light and pink by transmitted light. The inoculating strain and twelve isolates from tissues were tested in the following range of biochemical tests: catalase, oxidase, nitrate reduction, production of hydrogen sulphide in media with and without cysteine hydrochloride, growth in aerobic and anaerobic conditions, growth at 25 °C and 42 °C, and growth in the presence of nalidixic acid, triphenyltetrazolium chloride, brilliant green, glycine at 1% and 1·5%, sodium selenite and metronidazole.

Serology

Serum samples taken from the monkeys on two occasions before infection and on various occasions up to three months after infection were tested for antibodies against the inoculating strain by the following tests: tube agglutination, Coombs antiglobulin and complement fixation. In the Coombs test a commercial anti-human immunoglobulin preparation (Nordic Immunological Laboratories) was used. The antigens were prepared from the inoculating strain (V212 X) and included, for the agglutination and Coombs test, a whole cell suspension diluted in 0·25% formal saline to an opacity corresponding to Macfarland's tube 3 and for the complement fixation test, a sonicated whole cell suspension.

RESULTS

Clinical Findings

Four animals (A25, A31, A38, A40) were anorexic on the 4th and 5th days after infection, but the others continued to eat and drink normally throughout the course of the experiment. There was a significant pyrexia in six of the eight animals (A25, A26, A31, A33, A38, A40) on days 2, 3 and 5; temperatures of up to 105 °F were recorded (pre-infection and later values for the group were less than 103 °F). The faeces of three monkeys (A31, A34, A40) were very soft in consistency on day 4 and 5 and two of these monkeys (A34, A40) produced similar stools again on the 11th day. Three other monkeys (A21, A25, A26) had diarrhoea on the 3rd day only, and one of these (A25) developed a severe diarrhoea again on the 46th day, which persisted for 4 days. Two animals (A33, A38) did not exhibit diarrhoea at any stage. On challenge 15 weeks after initial infection the two recovered monkeys (A25 and A38) did not show any clinical signs of illness, had no pyrexia, and had normal stools for 44 days after re-infection.

Histopathology

Lesions attributable to the *C. jejuni* infection were not found at any stage in sections of the stomach or any region of the small and large intestines, or in the mesenteric lymph nodes, spleen, kidneys, or bladder. There was a low level of infestation by nematodes which was associated with the presence of occasional small foci of lymphoreticular cell infiltration in the lamina propria of the ileum, caecum, and colon.

There were, however, numerous foci of polymorphonuclear leucocytes (PMN) and lymphocytes in the liver of the monkeys killed at 7, 14, 30, and 46 days. There was necrosis of some liver cells in these foci. The foci were randomly scattered throughout the liver and had no consistent lobular distribution. The same specimens exhibited intense infiltration of the walls of bile ducts by PMN and lymphocytes, but in no animal were there lesions in the gall bladder. These lesions were not present in the livers of other monkeys from the same batch which were killed for other purposes and served as controls in the experiment.

Examination of sections of intestine stained by Gram and Warthin-Starry methods did not permit demonstration of campylobacter because large numbers of other organisms, such as spirochaetes, were also present, particularly in the caecum and colon.

Culture

The results of blood, faeces, and tissue culture are shown in Tables 1, 2, and 3 respectively. They indicate a brief period of bacteraemia followed by the prolonged presence of *C. jejuni* in faeces. The liver and gall bladder were the most consistently colonized sites. Monkey A34, killed 21 days after infection, did not give a positive culture from any tissue sampled. Nevertheless, this animal had definitely become infected, as shown by the isolation of *C. jejuni* from blood on day 1 and

Table 1. *Occurrence of* C. jejuni *in blood of infected monkeys*

Day after oral administration of 1×10^9 *C. jejuni*	Monkey					
	A21	A26	A31	A33	A34	A40
1	+	+ +	−	+ +	+	−
2	+	+ +	−	+	−	−
3	+ (K)	+ +	−	+	−	−
4	.	−	−	−	−	−
7	.	− (K)	−	−	−	−
8	.	.	−	−	−	−
9	.	.	−	−	−	−
11	.	.	−	−	−	+
14	.	.	−	− (K)	−	−
18	.	.	−	.	−	−
21	.	.	−	.	− (K)	−
24	.	.	−	.	.	−
30	.	.	−	.	.	− (K)
37	.	.	NT	.	.	.
46	.	.	− (K)	.	.	.

−, no *C. jejuni* detected; +, < 5 colonies; + +, 5 to 100 colonies; + + +, > 100 colonies; K, killed for bacteriological and histological examination; NT, no sample taken.

Table 2 *Occurrence of* C. jejuni *in faeces of infected monkeys*

Day after oral administration of 1×10^9 *C. jejuni*	Monkey					
	A21	A26	A31	A33	A34	A40
1	−	−	−	+	−	−
2	−	+	−	+	−	−
3	+ (K)	+	−	+	−	−
4	.	+	−	+	−	−
7	.	+ (K)	−	−	−	−
8	.	.	+	−	+	+
9	.	.	+	−	+	+
11	.	.	+	−	+	+
14	.	.	−	− (K)	+	
18	.	.	−	.	−	+
21	.	.	−	.	− (K)	+
24	.	.	+	.	.	+
30	.	.	+	.	.	+ (K)
37	.	.	+	.	.	.
39	.	.	−	.	.	.
43	.	.	+	.	.	.
46	.	.	− (K)	.	.	.

−, no *C. jejuni* detected; +, < 5 colonies; + +, 5 to 100 colonies; + + +, > 100 colonies; K, killed for bacteriological and histological examination.

faeces samples at intervals up to 14 days following infection (Tables 1 and 2). This animal had therefore completely eliminated the infection and recovered by day 21.

Twelve randomly selected isolates of *C. jejuni* from tissues and faeces, and the inoculating strain, gave identical results in the range of biochemical tests used (see Materials and Methods).

Table 3. *Occurrence of* C. jejuni *in tissues of infected monkeys*

Tissue	Day after infection* 3 (A21)	7 (A26)	14 (A33)	21 (A34)	30 (A40)	46 (A31)
Blood	+	−	−	−	−	−
Tonsil	−	+ + +	−	−	−	−
Stomach	+ + +	−	−	−	−	−
Duodenum	−	+ + +	+ +	−	+ +	−
Jejunum	−	−	−	−	+	−
Ileum	+ + +	−	+ +	−	+	−
Caecum	+ + +	−	+	−	+	−
Colon	+ + +	−	+	−	+	−
Rectum	−	−	−	−	+	−
Gall Bladder	−	+ + +	+ + +	−	+ + +	+
Liver	−	+ + +	+ +	−	+	−
Mesenteric lymph nodes	−	−	−	−	−	−
Spleen	−	−	−	−	−	−
Kidney	−	−	−	−	−	−
Bladder wall	−	+ +	−	−	−	−
Urine	not sampled	−	−	−	−	−
Faeces	+	+	−	−	+	−

* Animal codes are given in parenthesis.
−, no *C. jejuni* detected; +, < 5 colonies; + +, 5 to 100 colonies; + + +, > 100 colonies.

Table 4. *Culture of* C. jejuni *from blood and faeces of monkeys re-infected with* C. jejuni

Day after re-infection with 1×10^9 *C. jejuni*	Monkey A25 Faeces	A25 Blood	A38 Faeces	A38 Blood
1	+	−	−	−
2	+ +	−	+	−
3	+	−	+	−
4	−	−	−	−
5	−	NT	−	NT
7	−	NT	−	NT
11 to 44	−	NT	−	NT

−, no *C. jejuni* detected; +, < 5 colonies; + +, 5 to 100 colonies; + + +, > 100 colonies; NT, no sample taken.

Re-infection of monkeys with C. jejuni

The monkeys (A25 and A38) infected orally with 1×10^{10} *C. jejuni* showed *C. jejuni* intermittently in their faeces for a period of 45 days from the time of infection. Faeces were found to be negative for *C. jejuni* for a further period of 60 days. On this evidence the animals were assumed to be free from infection and were then given 1×10^9 of the same strain of *C. jejuni* orally. Table 4 shows the results of blood and faeces culture. There was no bacteraemia, such as resulted

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from primary infection, but for a period of three days *C. jejuni* was excreted in the faeces.

Serology

With one exception, none of the serum samples taken before or after inoculation gave significant titres to any of the serological tests. The exception was monkey A33 whose sample taken on day 10 post-inoculation gave a negative result to the tube agglutination test but was positive at titres of 160 in the Coombs test and 32 in the complement fixation test.

DISCUSSION

The experiments reported here show that infection of Rhesus monkeys with a human strain of *C. jejuni* is possible, although the clinical disease induced was milder than that seen in the children involved in the original outbreak from which this strain was isolated (Dr A. T. Willis, personal communication). Vomiting did not occur and the inappetence and diarrhoea were intermittent and of short duration. However, the Rhesus monkey did prove to be a suitable experimental animal for studying the excretion of the bacteria and their distribution in the body.

C. jejuni was isolated from faeces samples of the monkeys at varying times after oral infection. In two animals excretion was continuous for several days from day 1, but in others it did not commence until day 8. Thereafter excretion was intermittent, and in monkeys allowed to survive the faeces contained *C. jejuni* until the 43rd day after infection. After this time the infection appeared to have been completely eliminated. According to Butzler & Skirrow (1979), in untreated human infections, patients usually continue to excrete campylobacters for two to five weeks after an attack of enteritis. It is known that in man symptomless excretion of the organism may occur, and in one survey *C. jejuni* was reported in 5·3% of diarrhoeic stools and 1·6% of normal stools from both children and adults (Butzler, 1979).

Bacteraemia occurred in most monkeys for the period one to three days after infection. This supports the hypothesis of Butzler & Skirrow (1979) that bacteraemia is probably limited to the earliest stages of the disease. Despite bacteraemia there was only one isolation from organs not associated with the digestive system, even in the monkey killed at three days; this was the isolation from the bladder wall of the monkey killed at seven days. Infection of the urogenitial tract of monkeys naturally harbouring simian strains of *C. jejuni* has been recorded by Tribe & Frank (1980). It is of particular interest that the bacteria were never recovered from the mesenteric lymph nodes. This suggests that they may not invade lymphatics in the intestine and indicates a strikingly different pathogensis from diseases caused by some other invasive enteric pathogens, such as salmonella.

Histological examination of the different regions of the intestinal tract showed that, by light microscopy, no lesions could be detected at any stage of infection, even though organisms were cultured from adjacent sites, However, penetration through or between epithelial cells must have occurred in the first 24 h for the

bacteria to reach the blood vessels of the lamina propria or submucosa and appear in the peripheral blood. Electron microscopic studies by Butzler (1979) on experimentally infected poultry showed that *C. jejuni* is invasive and passes through the epithelial cells to gain access to the intestinal blood vessels. In the monkeys after the first three days of infection bacteraemia occurred only once, on day 11 in monkey A40. This may have been due to inhibition of penetration by the development at the intestinal surface of a local immune response or to circulating antibody 'mopping up' bacteria on arrival in the blood.

C. jejuni thus colonized principally the duodenum, ileum, caecum, colon, liver and gall-bladder, though in numbers which decreased with time. The stomach was positive only in the monkey killed on day three. The livers and bile duct walls had small foci of infiltration by PMN and lymphocytes which were associated with the presence of the bacteria. The liver foci were similar to those seen in salmonellosis in man and animals (Angrist & Mollov, 1948; Lawson & Dow, 1965; Anderson, 1966) though they were smaller and lacked the central area of necrosis of the typical typhoid nodule. With the exception of one monkey killed at 21 days, the gall-bladder contained *C. jejuni* at all stages from days 7–46. However, histological lesions were not seen in the gall-bladder and bacteria could not be detected in sections of gall-bladder stained by Gram or by the Warthin–Starry silver impregnation technique. Blaser *et al* (1980) and Darling *et al* (1979) reported that *C. jejuni* can survive in the liver and bile ducts for some weeks and pass in the bile into the gall bladder, and from this site be intermittently excreted into the intestinal lumen and faeces. These events might also occur in human infection, particularly in the case of symptomless excretors.

The largely negative humoral immune response was disappointing. This may have been due to the poor sensitivity of the test systems used. However, this is difficult to assess because there are, as yet, no widely standardized systems for campylobacter serology. The animal (A33) which did show a significant response was one of three which had had a fairly prolonged bacteraemia and, at necropsy it gave positive culture results from a number of sites. The other two monkeys which had also had prolonged bacteraemia were, by chance, the first two to be sacrificed – possibly before they had time to develop a detectable humoral antibody response.

The two monkeys challenged 15 weeks after their first infection (60 days after they ceased to excrete the organism in stool samples) resisted re-infection, as shown by the lack of clinical disease, the fact that bacteraemia did not develop, and the presence of *C. jejuni* in the faeces for only three days.

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