Pathological changes in the rabbit ileal loop model caused by *Campylobacter jejuni* from human colitis

P. H. EVEREST§, H. GOOSSENS*, P. SIBBONS†, D. R. LLOYD‡, S. KNUTTON‡, R. LEECE, J. M. KETLEY and P. H. WILLIAMS§

Department of Genetics, University of Leicester, Leicester LE1 7RH, *WHO Collaborating Centre for Enteric Campylobacter, Department of Microbiology, St Pieters Hospital, B-1000 Brussels, Belgium, †Department of Paediatric Surgery, Institute of Child Health, Great Ormond Street, London WC1N 1EH and ‡Institute of Child Health, University of Birmingham, Birmingham B16 8ET

**Summary.** Four strains of *Campylobacter jejuni* isolated from children with inflammatory diarrhoea were assayed in the rabbit ileal loop model of infectious diarrhoea. All caused inflammatory reactions with severe macroscopic and microscopic damage in infected rabbit ileal tissue similar to that observed in the patients by endoscopy and histological analysis of colonic biopsies. Haemoglobin and other proteins were observed in loop fluids, consistent with leakage of serum from damaged mucosa. Loop fluids also contained significant bicarbonate concentrations, indicative of an active secretory component similar to that in control loops inoculated with cholera toxin. However, although three of the four clinical strains produced small amounts of a protein immunologically related to cholera toxin, none such was detected in either tissues or fluids of infected ileal loops. We propose instead that host-derived mediators of secretion may be important in pathogenesis. A mutant strain of *C. jejuni* with impaired motility, obtained from the National Collection of Type Cultures, did not induce tissue damage or fluid secretion in rabbit ileal loops.

**Introduction**

*Campylobacter jejuni* is a major cause of enterocolitis among children and young adults in developed and developing countries. Symptoms range in severity from acute watery diarrhoea to those of an inflammatory disease that may be associated with tissue invasion and occasional bacteraemia. Several animal models that mimic the histopathology of human disease have been developed, but the animals used have generally been large and expensive, and no simple small mammal model for the pathogenesis of campylobacter enterocolitis exists that does not demand prior treatment or surgical procedures. The "removable intestinal tie adult rabbit diarrhoea" (RITARD) procedure has been used successfully for the analysis of histological changes and immune responses in *C. jejuni* infection, but secreted fluids are not available for analysis in this system, in which ligation of the ileum is only temporary. The rabbit ileal loop test (RILT) has also been used for various infectious diarrhoeal diseases, including a preliminary study of infection with *C. jejuni*. This technique has the advantage that both tissues and secreted fluids can be collected for histological and biochemical examination. The purpose of the work reported in this paper was to analyse the pathological effects of *C. jejuni* isolates from human colitis in the RILT, with a view to assessing its effectiveness as a model for the study of human disease.

**Materials and methods**

*Bacteria and culture conditions*

*C. jejuni* strains L115, C119, O81 and P71 used in this study have been described previously. They were isolated from the stools of children with inflammatory diarrhoea during bacteriological screening at the Department of Microbiology, St Pieters University Hospital, Brussels. Data from recto-colonoscopy, performed routinely to exclude ulcerative colitis and Crohn's disease, and histological analysis of colonic biopsies are shown in table I. All strains were motile as judged by phase contrast light microscopy, and all showed cytotoxic activity against cultured Chinese hamster ovary (CHO) cells. All except strain P71 produced cholera-like toxin, as previously determined by elongation of CHO cells, and confirmed here by monosialganglioside GM1-enzyme linked immunosorbent assays (GM1-ELISA). Mutant *C. jejuni* strain NCTC 12189 was also used; this strain has...
Table I. Histology in RILT compared with endoscopy and histology in man

<table>
<thead>
<tr>
<th>Inoculated strain or compound</th>
<th>Lesions in man</th>
<th>Histology</th>
<th>Histology in RILT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endoscopy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L115 (ENT+ CYT+)</td>
<td>Large nodular ulceration, severe oedema, hyperaemia, mucus and pus</td>
<td>Tissue oedema, cryptitis, marked PMNL infiltration</td>
<td>Blood-stained mucosa, flattened villi, cell damage, moderate PMNL infiltration, submucosal bleeding</td>
</tr>
<tr>
<td>C119 (ENT+ CYT+)</td>
<td>Large nodular ulceration, severe oedema, hyperaemia, mucus and pus</td>
<td>Tissue oedema, cryptitis, marked PMNL infiltration</td>
<td>Blood-stained mucosa, &quot;Christmas tree&quot; villi, moderate PMNL infiltration, submucosal oedema and bleeding</td>
</tr>
<tr>
<td>O81 (ENT+ CYT+)</td>
<td>Micronodular ulceration, moderate oedema, hyperaemia</td>
<td>Tissue oedema, marked PMNL infiltration</td>
<td>Haemorrhagic mucosa, damaged villi, marked PMNL infiltration, submucosal oedema and bleeding</td>
</tr>
<tr>
<td>P71* (ENT- CYT+)</td>
<td>Moderate hyperaemia</td>
<td>Moderate PMNL infiltration</td>
<td>Blood-stained mucosa, flattened villi, light PMNL infiltration, red cells in submucosa</td>
</tr>
<tr>
<td>NCTC 12189</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>Villi and submucosa normal</td>
</tr>
<tr>
<td>CT</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>Some &quot;Christmas tree&quot; villi, submucosa normal</td>
</tr>
<tr>
<td>PBS</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>Villi and submucosa normal</td>
</tr>
</tbody>
</table>

ENT, enterotoxin; CYT, cytotoxin; CT, cholera toxin.
PMNL, polymorphonuclear leucocytes.
*Loops inoculated with P71, which showed no fluid, also showed no histological damage.

reduced motility, despite retaining intact flagella, and is unable to colonise the intestinal tract of infant mice.24 Bacteria were grown micro-aerophilically in Mueller-Hinton (MH) broth overnight at 42°C,5 harvested by low speed centrifugation, and re-suspended at c. 10^8 bacteria/ml in phosphate-buffered saline (PBS) for injection into rabbit ileal loops.

Rabbit ileal loop tests

Surgical procedures on 7–9 week-old specific pathogen-free New Zealand White rabbits (< 2 kg) were performed under licence by standard technique.17,18 Briefly, laparotomy was performed on anaesthetised animals from the lower liver margin to the level of the iliac fossa. The distal ileum and the ileo-caecal junction were elevated, and 5-cm long sections of ileum, 10 cm apart, close to the ileo-caecal junction, were tied with silk thread. Two animals were used for each bacterial strain, each animal having two test loops and two control loops. Test loops were inoculated with 0.5-ml volumes of bacterial suspensions in phosphate-buffered saline (PBS). Positive control loops were inoculated with 1 µg of cholera toxin (CT) in 0.5 ml of PBS; negative control loops received 0.5 ml of PBS alone. Loops were replaced in the peritoneal cavity in their original positions, and the peritoneum was closed. Animals were again anaesthetised 18 h after inoculation, and laparotomy was performed as before. Loops were removed and weighed, and loop fluids were stored in sterile containers. Loop tissue was washed in PBS and fixed in formaldehyde 3%; thin sections were stained with haematoxylin and eosin, and examined under bright field illumination with a Zeiss Axiophot microscope. Homogenised tissue extracts were tested for the presence of cholera-like enterotoxin by GM1-ELISA (see below).

Fluid analysis

Loop fluids were centrifuged to remove cellular components and assayed for bicarbonate (mm), pH and haemoglobin (mg/ml) with a Howe ABL-2 acid-base laboratory analyser (Radiometer, Copenhagen), and for total protein (mg/ml) by the method of Bradford.25 Fluids were also examined by bright field microscopy for the presence of red and white blood cells, and tested for cholera-like enterotoxin by GM1-ELISA (see below).

Microbiological analysis

Loop fluids were plated for viable counts of C. jejuni on MH agar essentially as described by Miles et al.26 Loop tissue samples (c. 0.5 g) were washed with PBS, homogenised in 0.5 ml of PBS with a pestle and mortar, and plated for viable counts on MH agar. Blood samples taken post mortem were lysed with deoxycholate (Sigma) 0.5% and plated for viable counts on MH agar. Peritoneal fluids and homogenised samples (c. 0.5 g) of liver and gall bladder tissue taken post mortem were cultured on MH agar for C. jejuni. Bacterial serum sensitivity was determined as described by Taylor.35 Briefly, (2–5) x 10^8 viable bacteria in gelatin–veronal buffer containing
calcium and magnesium were incubated at 37°C in a 1 in 25 dilution of human or rabbit serum. Samples taken at intervals over a period of 3 h were subjected to viable counts on MH agar.

GM1-ELISA for cholera-like enterotoxin

GM1-ELISA was performed essentially as previously described. Wells of 96-well microtitration
identical with that observed (figure, although strain C119 produced small amounts of intestinal tissue infected with strain C119, some of the villi resembled Christmas trees (figure, e), a response culture supernates morphonuclear leucocyte (PMNL) infiltrate with macrophages (figure, b), tissue oedema (figure, c), and cell damage and submucosal bleeding (figure, d). In cholera-like enterotoxin, as determined both by CHO producing inflammatory strains, L115 and 08 1, which cell elongation6 and by GM1-ELISA of MH broth haemorrhage; one loop inoculated with strain 081 isolated from those patients (table I). Some loops forated. Tissue sections typically indicated inflam- and one loop inoculated with strain P71 were per- showed macroscopic damage with obvious mucosal hstopathological changes in colitis patients and in ligated rabbit ileal loops infected with C.

Histological effects of C. jejuni infection

Clear similarities were observed between the severity of histopathological changes in colitis patients and in ligated rabbit ileal loops infected with C. jejuni strains isolated from those patients (table I). Some loops showed macroscopic damage with obvious mucosal haemorrhage; one loop inoculated with strain 081 and one loop inoculated with strain P71 were perforated. Tissue sections typically indicated inflammatory reactions comprising predominantly polymorphonuclear leucocyte (PMNL) infiltrate with macrophages (figure, b), tissue oedema (figure, c), and cell damage and submucosal bleeding (figure, d). In intestinal tissue infected with strain C119, some of the villi resembled Christmas trees (figure, e), a response identical with that observed (figure, f) and previously reported27 for CT-mediated fluid secretion. However, although strain C119 produced small amounts of cholera-like enterotoxin, as determined both by CHO cell elongation6 and by GM1-ELISA of MH broth culture supernates (0.003 μg/ml), no activity was detected by GM1-ELISA in homogenised tissue samples infected with strain C119 (< 0.0001 μg/ml). Similar results were obtained for the two enterotoxin-producing inflammatory strains, L115 and 081, which did not elicit Christmas tree-like villous morphology. However, the possibility that toxin bound to or inside cells was not released by tissue homogenisation cannot be ruled out, although CT was detected by GM1-ELISA in CT-treated loop tissue (0.02-0.14 μg/ml). Strain NCTC 12189 elicited no histological changes in the rabbit mucosa.

Loop fluid analysis

All four inflammatory isolates stimulated fluid accumulation in infected loops, but fluid volumes were lower than those in loops inoculated with CT. For strains L115 and C119, all four test loops contained fluid. One of the four loops infected with strain 081 had ruptured, but fluid was recovered from the other three loops. Strain P71, which was associated with less severe symptoms in patients (table I), caused fluid accumulation in only two loops, one in each test animal, but fluid was lost by rupture of one of the loops. Fluids from infected loops did not have the appearance of frank blood, but in all cases macroscopic or microscopic blood was observed, and PMNLs were present, suggesting tissue damage. Consistent with this, haemoglobin and other protein concentrations were higher in loop fluids induced by C. jejuni infection than in those from CT-treated loops, in which fluid was induced solely by an active secretory process (table II). Fluids induced by bacterial infection contained generally higher bicarbonate concentrations than normal rabbit blood (16.2-31.8 mM); generally, higher fluid bicarbonate concentrations correlated with higher pH (table II). High bicarbonate concentrations were also observed in fluids stimulated by CT treatment. Cholera-like enterotoxin was not detected by GM1-ELISA in loop fluids induced by inflammatory strains that produced small amounts of toxin in vitro (< 0.0001 μg/ml), although the method detected CT in the fluids of CT-treated loops (0.04-0.5 μg/ml). No fluid accumulation was observed with strain NCTC 12189.

Table II. Fluid production and analysis

<table>
<thead>
<tr>
<th>Inoculated strain or compound</th>
<th>Positive loops (total inoculated)</th>
<th>Mean fluid volume, ml (range)</th>
<th>Mean bicarbonate mm (range)</th>
<th>pH</th>
<th>Protein content, mg/ml (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>total</td>
</tr>
<tr>
<td>L115</td>
<td>4/4</td>
<td>30</td>
<td>(197-628)</td>
<td>7.83</td>
<td>2.0</td>
</tr>
<tr>
<td>C119</td>
<td>4/4</td>
<td>82</td>
<td>(156-938)</td>
<td>7.92</td>
<td>2.0</td>
</tr>
<tr>
<td>O81</td>
<td>4/4*</td>
<td>(1-5)</td>
<td>(7.53-14)</td>
<td>2.5</td>
<td>0.35</td>
</tr>
<tr>
<td>P71</td>
<td>2/4*</td>
<td>50</td>
<td>(17.7-43)</td>
<td>8.08</td>
<td>5.0</td>
</tr>
<tr>
<td>NCTC 12189</td>
<td>0/4</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CT</td>
<td>10/10</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>0.34</td>
</tr>
<tr>
<td>PBS</td>
<td>0/10</td>
<td>0</td>
<td>6.5†</td>
<td>7.27†</td>
<td>0†</td>
</tr>
</tbody>
</table>

Values are averages for loops from which fluid was recovered. ND, not done, no fluid secretion. *Fluid was lost due to perforation of one positive loop inoculated with strain O81 and one inoculated with P71. †Values for PBS before injection into loops.
Microbiological analysis

Extensive mucosal colonisation by strains L115, C119 and O81 was indicated by high viable bacterial counts in samples of infected loop tissue and, consequently, in loop fluids (table III). Strain P71 colonised to a lesser extent, but nevertheless caused significant tissue damage. Viable bacterial counts of strain NCTC 12189 in loop tissue were similar to those of P71, but colonisation with the mutant strain was not associated with tissue damage in this model. Post-mortem blood cultures were positive for all strains except NCTC 12189, suggesting true bacteraemia resulting from tissue damage, rather than operational trauma. However, viable counts were very low (10–12 bacteria/ml of blood) presumably because strains were sensitive to the bactericidal action of serum, both before and after loop inoculation (table III). The peritoneal fluids of all animals infected with the four inflammatory strains of C. jejuni were culture positive, as were the liver and gall bladder of rabbits infected with strain L115 (data not shown). This probably reflects seeding from the bloodstream.

Discussion

Studies with the RITARD technique indicate that the rabbit ileum is a good model for histopathological changes associated with campylobacter enterocolitis in man. The particular advantage of the RITARD method is that infection can be initiated by the oral route, the usual route of natural infection in man; its main disadvantage is that it is not possible to assess the nature of fluid secretion in conjunction with tissue damage. The RILT, on the other hand, does not allow oral infection but has the advantage that the effects of variability between animals can be minimised by the inclusion of control loops alongside test loops. This also reduces the number of animals needed. Important, too, is the fact that intestinal fluids can be recovered for analysis.

Strains of C. jejuni isolated from cases of human colitis differed somewhat in their effects in this model. Those that caused more severe disease in man, as judged by endoscopy and histological examination, caused greater histological damage in rabbit ileal loops, but all four produced shortened villi, white cell infiltration and bleeding into the mucosa, observations consistent with the inflammatory type of illness seen in man, in which faecal samples typically contain blood and PMNLs. Bacteraemia was observed with all four clinical isolates in this study. Campylobacter bacteraemia in man is not as rare as was previously suspected, particularly among hospitalised patients. However, since most C. jejuni strains are sensitive to the bactericidal action of normal serum, isolation of organisms from blood cultures presumably reflects their presence within circulating white cells.

Biochemical analysis of loop fluids induced by C. jejuni infection is consistent with leakage of serum proteins and blood cells into the gut lumen through damaged epithelial and endothelial tissues, as reported for other organisms. It is tempting to speculate that cytotoxins are the cause of this, since all four strains produced detectable cytotoxic activity. However, antibodies against cytotoxins have not been reported in convalescent sera, and their role in natural disease remains in doubt. Our results also indicate the involvement of an active secretory component in fluid accumulation, reflected in bicarbonate concentrations greater than would be expected due to serum leakage alone. Such bicarbonate loss is typical of infectious diarrhoeal diseases such as cholera; however, we obtained no evidence for the involvement of a cholera-like enterotoxin in fluid secretion induced by inflammatory strains of C. jejuni in this model. This is consistent with previous suggestions that there is no obvious association between toxin production and the type of clinical illness observed. Moreover, human volunteer studies indicate that enterotoxin alone cannot explain the clinical features of the inflammatory type of disease observed in developed countries, and antibodies against enterotoxin have not been reported in convalescent patients.

Therefore, since cholera-like enterotoxin is unlikely to be an important component of the pathogenesis of C. jejuni-induced diarrhoea, we propose instead the
involvement of host-derived mediators of secretion associated with tissue inflammation, as has been suggested for Salmonella typhimurium and Shigella flexneri. The nature of the host secretory response in C. jejuni-induced inflammatory diarrhoea is currently under investigation in our laboratories.

References